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Usage of Antimicrobials on 60 Dairy Farms in Northern Germany and Characterization of Methicillin-Resistant *Staphylococcus aureus* (MRSA) and Extended Spectrum Beta-Lactamases Producing *Escherichia coli* (ESBLs-producing *E. coli*) Isolated from Bulk Tank Milk Samples

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Chapter 1
Introduction

Since their discovery, antimicrobial agents have been widely used to treat infectious diseases in man and animals. Furthermore, in food producing animals, these drugs have been used not only in the treatment of infectious diseases, but also in order to prevent diseases (prophylactic use), to limit the spread of bacterial diseases (metaphylactic use) as well as to promote growth (growth promotors) (McEwen and Fedorka-Cray, 2002; Aarestrup, 2005). The occurrence of crowding-associated infectious diseases such as pneumonia or diarrhea is related to increasing numbers of animals being kept on one site and results in an increased use of antimicrobials in the latter animals. This practice bears the risk for the emergence of resistant bacteria by selection of such bacteria or occurrence of resistant mutants (McEwen and Fedorka-Cray, 2002; Aarestrup, 2005; Dancer, 2008; Bergman et al., 2009).

As there are no agreements on the use of distinct antimicrobial agents in either humans or animals, similar antimicrobial substances are used in human and veterinary medicine (Schwarz et al., 2001; McEwen and Fedorka-Cray, 2002). The prudent antibiotic use by veterinarians is an important tool to reduce the number of antibiotic treatments in veterinary practice in order to avoid the development of resistance (Ungemach et al., 2006).

The increasing prevalence of antimicrobial resistant bacteria is a major concern in human and veterinary medicine. Methicillin-resistant *Staphylococcus aureus* (MRSA) and extended-spectrum beta-lactamases producing *Escherichia coli* (ESBLs-producing *E. coli*) are the main bacterial species in which resistance is of greatest public health concern (WHO, 2011b). MRSA has been known for a long time as a major threat of human health due to its resistance with respect to antibiotics routinely used in the treatment of bacterial infections in man. In the United States, the number of people developing a serious MRSA infection in 2005 was estimated 94,360, with a fatal outcome in 18,650 patients (Klevens et al., 2007). In Germany, the prevalence of MRSA positive from *S. aureus* infected patients increased from < 2% in 1990 to > 20% in 2001 (GERMAP, 2008). *E. coli* is a frequent cause of urinary tract infections in patients of hospitals in Germany, and it accounts for 15% of the cases of nosocomial bacteremia. For *E. coli*, increasing rates of resistance were recorded with respect to antimicrobials, and increased resistance against the groups of third and fourth generation cephalosporins can be explained in part by the emergence of ESBLs-

producing organisms (GERMAP, 2008). The European Antimicrobial Resistance Surveillance Network (EARS-Net) reported that in 22 countries participating in EARS-Net the proportion of 3rd generation cephalosporin-resistant *E. coli* increased significantly (European Centre for Disease Prevention and Control, ECDC, 2010)

In dairy cattle, antimicrobial agents are used for different reasons: In first instance to treat animals suffering from bacterial infections, mainly of the respiratory (pneumonia), digestive (diarrhea), reproductive (metritis) tracts and of the udder (mastitis), in second instance to prevent disease in the healthy animal e.g. by intramammary instillation of antimicrobials in dry cow treatment (Aarestrup, 2005). The use of antimicrobials in dairy cattle bears potential risks for human health when the final product derived from the animal contains residues of antimicrobials or even live resistant bacterial strains (McEwen et al., 1991; Ruegg and Tabone, 2000; Ruegg, 2005).

S. aureus is one of the major contagious mastitis pathogens in dairy cows, and the first isolations of MRSA from animals were in milk from cows with mastitis. Recently, the study of MRSA in US bulk tank milk reported that 218 bulk tank milk samples (40.2%) were positive for *S. aureus*, but none were positive for MRSA (Virgin et al., 2009). *E. coli* are gram negative, rod-shaped bacteria, which are inhabitants of the gastrointestinal tract of warm-blooded animals. *E. coli* is one of the most important environmental mastitis pathogen in dairy cows. Many studies on antimicrobial resistant *E. coli* in dairy cattle have been reported, with most of the latter focusing on *E. coli* isolated from faecal samples (Berg et al., 2005; Donaldson et al., 2006; Lundin et al., 2008). Only a few studies have been performed on antimicrobial-resistant *E. coli* isolated from bulk tank milk samples (Straley et al., 2006; Berg et al., 2007). The source of *E. coli* in bulk tank milk, however, was assumed to be a result of faecal contamination (Jayarao and Wang, 1999).

Milk could be a source of MRSA and ESBLs-producing *E. coli*, and for this reason might serve as a reservoir of antimicrobial resistance determinants. For this reason the aims of the present study are as follows:

- Find out if MRSA and ESBLs-producing *E. coli* are present in bulk tank milk samples obtained from different dairy farms in northern Germany
- Characterization of MRSA isolated from bulk tank milk samples
- Investigation of the risk factors associated with ESBLs-producing *E. coli* positives in bulk tank milk

Chapter 2
Review of literature

2.1 Antimicrobials and antimicrobial resistance

Antimicrobial agents are substances of natural, semi-synthetic, or synthetic origin that at *in vivo* concentrations kill or inhibit the growth of micro-organisms by interacting with a specific target but cause little or no damage to the host (FAO/WHO/OIE, 2008). In human medicine, antimicrobial agents have successfully been used for the last 70 years in the treatment of patients suffering from infectious diseases of bacterial origin. The outcome of the treatment with antimicrobials greatly depends on the duty of care the patients demonstrate with respect to the doctor's prescription.

Antimicrobials have widely been used for the treatment of animals suffering from bacterial infections. In food producing animals these drugs have been used for therapeutic, prophylactic and metaphylactic reasons as well as to promote growth by exerting their effects on the intestinal microbial flora (Aarestrup, 2005). The use of antimicrobials in humans and animals, however, bears certain risks. Any use of antibiotics in human and veterinary medicine can lead to the development of resistant microorganisms. Resistance emerges when a microorganism mutates or acquires a resistance gene. Some microorganisms may develop resistance with respect to a single antimicrobial agent or a distinct class of antimicrobial agents, while others develop resistance with respect to several antimicrobial agents or classes. These organisms are often referred to as multidrug-resistant or MDR strains. In some cases, the microorganisms have gained an antimicrobial resistance pattern directed at most of the available antibiotics.

Antimicrobial resistance is a global public health issue with impact on both human and non-human antimicrobial usage. The continuing emergence, development and spread of pathogenic organisms that are resistant to antimicrobials are a cause of increasing concern. World Health Organization (WHO, 2011a) stated "People infected with resistant microorganisms often fail to respond to conventional treatment, resulting in prolonged illness and greater risk of death. Antimicrobial resistance in microorganisms hampers the control of infectious diseases by reducing the effectiveness of treatment, which increases the period of time infected patients shed the infectious agent, potentially spreading the latter agent among contact persons. Furthermore, the emergence of resistant microorganisms increases the costs for therapeutic interventions due to the increased duration of treatment

and the need for the use of innovative - thus more expensive - antimicrobials. The longer duration of illness and treatment, often in hospitals, increases health-care costs and the financial burden to families and societies".

Several microorganisms which cause infections in humans and animals have developed resistance directed at one or even more than one antimicrobial. Some of the bacterial species in which antimicrobial resistance is of greatest public health concern are given below (WHO, 2011b):

Bacteria - Community

- *Escherichia coli*
- *Mykobacterium tuberculosis* (cause of tuberculosis)
- *Neisseria gonorrhoeae* (cause of gonorrhoea)
- *Salmonella typhi*
- *Staphylococcus aureus*, including community-associated MRSA (Methicillin-Resistant *S. aureus*)
- *Streptococcus pneumoniae*

Bacteria – Hospitals

- *Acinetobacter baumannii*
- *Enterococcus faecium* and *Enterococcus faecalis*, including VRE (Vancomycin-resistant enterococci)
- Multidrug-resistant enteric pathogens, including *Escherichia coli* and *Klebsiella pneumoniae* producing ESBL (Extended-spectrum Beta-Lactamase) and KPC (Klebsiella Pneumonia Cabapenemase) enzymes
- *Pseudomonas aeruginosa*
- *Staphylococcus aureus*, including MRSA (Methicillin-Resistant *S. aureus*)
- *Stenotrophomonas maltophilia*

Bacteria - Zoonotic disease

- *Campylobacter* species
- *Salmonella* species

2.2 Antimicrobial resistance

Antimicrobial resistance is the ability of a microorganism to multiply or persist in the presence of increased levels of an antimicrobial agent relative to the susceptible counterpart of the same species (FAO/WHO/OIE, 2008). Normally, susceptible populations of bacteria may become resistant to antimicrobial agents through mutation and selection. Strains of bacteria carrying resistance genes originating from mutations are selected by the effects of antimicrobials used for therapeutic reasons, which kill the susceptible strains but allow the newly resistant strains to survive and multiply. Resistance that develops due to chromosomal mutation and selection is termed **vertical evolution**. Bacteria also develop resistance by acquiring from other bacteria the genetic information encoding for resistance. This process is termed **horizontal evolution**, and may occur between strains of the same species or between different bacterial species or genera. Of greater concern are cases of acquired resistance which may occur through the transfer of extra-chromosomal mobile genetic elements such as plasmids, integrons, and transposons. The extra-chromosomal mobile genetic elements may be transferred between bacteria by conjugation (cell to cell contact), transduction (bacteriophage introduction) or transformation (uptake of naked DNA) (Schwarz and Chaslus-Dancla, 2001; White and McDermott, 2001; Tenover, 2006). Conjugation is considered to be most important way for the spread of resistance genes (Schwarz and Chaslus-Dancla, 2001).

2.3 Therapeutical use of antimicrobials and first reports on resistance development

Most of antimicrobial agents currently used in human and veterinary medicine are low molecular weight substances which inhibit growth of bacteria or even kill them at very low concentrations. The first antimicrobials used represented substances or close relatives of substances which were produced by fungi or soil bacteria and provided a selective advantage to the antimicrobial producer in the fight for resources and ecological niches. Thus bacteria have come into contact with antimicrobial substances a long time before the first antimicrobial agents were used as therapeutics (Schwarz and Chaslus-Dancla, 2001). As a result of the exposure of bacteria to antimicrobial agents, a large number of resistance genes have developed.

Table 1. Overview over the year of discovery of various antimicrobial agents, the first therapeutical use and first reports on resistance development (adapted from EMEA, 1999)

Antimicrobial agent	Discovered	The first therapeutical use	Resistance identified
Penicillin	1940	1943	1940
Streptomycin	1944	1947	1947, 1956
Tetracycline	1948	1952	1956
Erythromycin	1952	1955	1956
Vancomycin	1956	1972	1987
Nalidixic acid	1960	1962	1966
Gentamicin	1963	1967	1970
Fluoroquinolones	1978	1982	1985

Table 1 shows the time coincidence between the introduction of antimicrobial agents into clinical use and the first reports on the occurrence of bacteria which are resistant to these particular substances.

2.4 Resistance mechanisms

Generally, resistance to antimicrobial agents evolves by five major mechanisms (White and McDermott, 2001):

1. Change in cell membrane permeability that prevents access of antimicrobials into the bacterium
2. Enzymatic inactivation or destruction of the antimicrobials
3. Alteration of the target site of antimicrobial action
4. Active efflux of antimicrobials out of the bacterium which prevent accumulation of antimicrobials within the cell
5. Creation of altered enzymatic pathways around those targeted by the antimicrobials

2.5 Methicillin-resistant *Staphylococcus aureus* or MRSA

Staphylococcus aureus is a gram-positive coccus, which is gathering in grape-like clusters when viewed through a microscope. It grows in large, round, golden-yellow colonies, often

with hemolysis, when grown on blood agar. *S. aureus* is well recognized as pathogen in both human and veterinary medicine. In human medicine, *S. aureus* is a potentially pathogenic bacterium that can cause various diseases ranging from minor infections of the skin to post-operative wound infections, bacteremia, necrotizing pneumonia, and nosocomial infections. In animals, *S. aureus* is an important cause of mastitis in dairy cattle and skin and soft tissue infections in food producing animals and companion animals and horses.

Methicillin resistant *Staphylococcus aureus* or MRSA is a bacterium causing severe infections in humans (mainly hospitalized), which – due to its wide resistance spectrum are difficult to treat. MRSA is a strain of *S. aureus* that is resistant to a large group of antibiotics called the beta-lactams, which include penicillins and cephalosporins. It has evolved an ability to survive treatment with beta-lactamase resistance beta-lactam antibiotics including methicillin, dicloxacillin, and oxacillin. Resistance of MRSA to all beta-lactam antibiotics is mediated by a *mec*A gene that encodes the production of an altered penicillin-binding protein (PBP2a). The *mec*A gene is located on a mobile genetic element called the Staphylococcal Cassette Chromosome *mec* (SCC *mec*). Therefore, beta-lactam antibiotics are not effective against MRSA because these drugs cannot bind to the bacterial cell wall.

In dairy cattle, *S. aureus* is a major cause of chronic or recurring clinical mastitis, and is regarded as a major contagious mastitis pathogen. In the Republic of Korea, 21 *S. aureus* strains (2.5%) from bovine mastitic milk samples were resistant to methicillin (Moon et al., 2007). Many of *S. aureus* strains are resistant to penicillin or ampicillin because of long-term use of beta-lactam antibiotics in agricultural and healthcare settings, and recently, *S. aureus* generally exhibits a multiple resistance to antimicrobial drugs such as tetracyclines, aminoglycosides, macrolides, and lincosamides (Moon et al., 2007).

2.6 MRSA strains

Different strains of MRSA have been identified based on phenotypic and genotypic analysis. MRSA nomenclature varies worldwide, and a standard method for typing and naming MRSA strains has not yet been adopted. Therefore, one genetic strain of MRSA may be referred to by several different names in various scientific papers. MRSA strains can be typed by both, phenotypic and molecular methods. Phenotypic methods include: colonial characteristics, biochemical reactions, antibiotic susceptibility patterns, and the susceptibility to various phages and toxin production. Molecular typing methods include

pulsed field gel electrophoresis (PFGE), multilocus sequence typing (MLST), *SCCmec* and *spa* typing.

2.6.1 Healthcare-Associated MRSA or Hospital-Associated MRSA (HA-MRSA)

MRSA are regarded as HA-MRSA when infections caused by these pathogens are likely to be acquired in human health care setting. HA-MRSA appear at least 48 h after admission of a patient to a hospital, in particular when certain risk factors are present such as risk of nosocomial acquisition of infection including prolonged antimicrobial therapy, surgery, prolonged hospital stay, treatment in an intensive care unit and close proximity to other patients infected or colonized with MRSA.

2.6.2 Community-Associated MRSA (CA-MRSA)

CA-MRSA infections occur in healthy people without history of hospitalization or medical procedures, and are usually associated with skin and soft tissue infections. Close contact, crowding, contaminated surface and shared items, as well as poor hygienic conditions in sport facilities, schools, day care centers, military settings, and prisons, are considered risk factors.

2.6.3 Animal-Associated MRSA (AA-MRSA) or Livestock-Associated MRSA (LA-MRSA)

Strains of MRSA have been recently discovered which are harboured by animals; these strains are termed Animal-Associated MRSA (AA-MRSA) or Livestock-Associated MRSA (LA-MRSA) or Non-Typable MRSA (NT-MRSA). LA-MRSA refers mainly to the clonal spread of a certain MRSA strain (ST398) that colonizes different food animal species and may cause infections in humans.

2.7 MRSA Prevalence

2.7.1 MRSA in humans

Methicillin-resistant *Staphylococcus aureus* (MRSA) has been known for a long time as a major human health problem due to its resistance to most traditional antibiotics. Originally, human MRSA infections were only found in hospitals (HA-MRSA), but nowadays, MRSA

infections are increasingly observed in the general community (CA-MRSA) (Elston and Barlow, 2009).

Reports from the United States from the year 2005 give an estimation of the number of people who developed a serious MRSA infection being 94,360; approximately 18,650 persons died from this infection during a hospital stay (Klevens et al, 2007). In Germany, the prevalence of MRSA positive from *S. aureus* infected patients increased from < 2% in 1990 to > 20% in 2001. As the vast majority of MRSA displays a multiresistance phenotype, this increase has not only limited the use of beta-lactams but also that of other drug classes, in particular fluoroquinolones (Ciprofloxacin), macrolides (erythromycin) and clindamycin (GERMAP, 2008).

2.7.2 MRSA in companion animals and horses

Few data on MRSA prevalence in animals are available. Identification of colonized and infected animals is important in the prevention of the spread of MRSA. Up to now the prevalence of MRSA has been reported for companion animals (dogs,cats,horses) (Table 2) as well as for food-producing animals (cattle, pigs, poultry).

Dogs and cats

MRSA has been isolated from dogs in Europe, Asia, and Australia (Kwon et al., 2006; Malik et al., 2006; Rich and Roberts, 2006; Walther et al., 2008). Prevalence of MRSA in clinical diseased dogs was 7.5% in Germany (Walther et al., 2008) and 2% in Korea (Kwon et al., 2006). With regard to MRSA carriage, a yearlong survey, including 6519 samples from healthy dogs submitted to a UK diagnostic laboratory, identified 95 MRSA isolates (0.4%) (Rich and Roberts, 2006). In Germany, the prevalence of MRSA in clinical infections in cats was 10% (Walther et al., 2008) but MRSA was not isolated from any of 12 hospitalized cats in a referral small animal hospital in the UK (Loeffler et al., 2005).

Horses

In a study by Cuny et al.(2006) including horses admitted to the Vienna Veterinary University Hospital between 2006 and 2007, MRSA positive strains were found in 25 of 140 horses with wound infections (either upon admission or as a result of post-surgical complications). In the UK, the prevalence of MRSA in diseased and clinically healthy

horses was 4.4% and 16%, respectively (Baptiste et al., 2005). In Belgium, 10.9% of horses presented at a veterinary hospital were found MRSA positive (Van den Eede et al., 2009).

Table 2. Prevalence of MRSA in companion animals including horses (adapted from EFSA, 2009)

Species/healthy or clinical disease	Year	Country	Prevalence of MRSA	Reference
Dogs/clinical	2003-2004	Germany	7.5%	Walther et al., 2008
Dogs/clinical	2001-2003	Korea	2%	Kwon et al., 2006
Dogs/healthy	2003-2006	UK	0.4%	Rich and Roberts, 2006
Dogs/clinical and healthy	Not given	UK	8.9%	Loeffler et al., 2005
Dogs/healthy	Not given	Australia	0%	Malik et al., 2006
Cats/clinical	2003-2004	Germany	10%	Walther et al., 2008
Cats/clinical	Not given	UK	0%	Loeffler et al., 2005
Horses/clinical	Not given	UK	4.4%	Baptiste et al., 2005
Horses/healthy	Not given	UK	16%	Baptiste et al., 2005
Horses/clinical	2003-2005	Austria	1.2 - 5.5%	Cuny et al., 2006
Horses/healthy	2004	The Netherlands	0%	Busscher et al., 2006
Horses/presented at vet. hospital	2007	Belgium	10.9%	Van den Eede et al., 2009
Horses/healthy	2006	Canada	0%	Burton et al., 2008
Horses/clinical and healthy	2006-2007	Canada	0.8 – 2.7%	Tokateloff et al., 2009

In a recent study at the Veterinary Microbiological Diagnostic Center, Utrecht University, the Netherlands, the percentage of MRSA isolates found in equine clinical samples increased from 0% in 2002 to 37% in 2008, and MRSA of *spa*-type t064, belonging to MLST ST8 and *spa*-types t011 and t2123, both belonging to the livestock-associated MLST ST398, predominated (van Duijkeren et al., 2010). During an outbreak of post-surgical MRSA infections in horses at a veterinary teaching hospital in 2006/2007, MRSA isolates were cultured from 7 horses and four out of 61 staff members which indicated

zoonotic transmission (van Duijkeren et al., 2010). However, another outbreak occurred in 2008, where 17 equine MRSA isolates were detected and 16 out of 170 staff members were found positive for MRSA (van Duijkeren et al., 2010). Personnel in close contact with horses were more often MRSA-positive (15/106) than those without (1/64) (van Duijkeren et al., 2010). Moreover, the latter study showed that 9.3% of horses were MRSA-positive, when they were sampled at admission; weekly cross-sectional sampling of all hospitalized horses for 5 weeks showed that 42% of the horses were MRSA-positive for at least one occasion, which suggests that nosocomial transmission took place during hospitalization. 53% of the environmental samples were found MRSA-positive, including samples from students' and staff members' rooms. This indicates that humans contribute to spreading of the organism. In a survey conducted in Canada, MRSA colonization was not identified in any of 497 healthy horses from Atlantic Canada (Burton et al., 2008), but was present in 1.1% of horses from Saskatchewan, 0.8% from Alberta, and 2.7% from British Columbia (Tokateloff et al., 2009).

2.7.3 MRSA in food producing animals

Studies on the prevalence of MRSA in food producing animals, including pigs, poultry and cattle, in several countries in Europe, Canada, and Asia are shown in Table 3.

Cattle

Staphylococcus aureus is one of the major contagious mastitis pathogens in dairy cows. The first isolations of MRSA from animals were reported for milk samples obtained from cows with mastitis. Therefore, many studies have been carried out on milk samples from dairy cows. These studies were carried out on a herd–based level on five dairy farms (one located in the Netherlands, four in Belgium) (Vicca et al., 2008). The percentage of MRSA positive cows in the latter herds varied from 0 to 14.3%. Moreover, Vanderhaeghen et al. (2010) reported that the *mec*A gene was detected in 11 (9.3%) of the 118 isolated *S. aureus* strains originating from 118 different farms in Belgium, indicating that nearly 10% of these farms have an MRSA problem. In Korea, 21 *S. aureus* strains (2.5%) derived from milk samples of cows with mastitis were MRSA positive (Moon et al., 2007). On a dairy farm in Hungary, Juhasz-Kaszanyitzky et al. (2007) found MRSA in 27 cows with subclinical mastitis and in one staff member of the farm. This finding indicated the transmission of MRSA between cows and humans. Additionally, in the recent study of

methicillin-resistant *Staphylococcus aureus* ST398 in veal calf farming in the Netherlands found that the prevalence of MRSA isolated from nasal swabs was 28% in veal calves, 33% in farmers, and 8% in family members, and the results from this study shows the direct associations between animal and human carriage of MRSA ST398 (Graveland et al., 2010).

Table 3. Prevalence of MRSA in food producing animals (adapted from EFSA, 2009)

Animal species	Year	Country	Prevalence of MRSA	Reference
Pig	2005-2006	The Netherlands	39%	De Neeling et al., 2007
Pig	2006	The Netherlands	11%	Van Duijkeren et al., 2008
Pig	Not given	Canada	25%	Khanna et al., 2008
Pig	2005	Denmark	10%	Guardabassi et al., 2007
Pig	Not given	Germany	49 – 70.8%	Tenhagen et al., 2009
Calves	2007-2008	The Netherlands	88% veal calf farms, 28% calves	Graveland et al., 2010
Dairy cows	1997-2004	Korea	19/696 mastitis isolates	Moon et al., 2007
Dairy cows	2002-2004	Hungary	27/595 mastitis isolates	Juhasz Kaszanyitzky et al., 2007
Dairy cows	2006	Belgium and The Netherlands	0 – 14.3%	Vicca et al., 2008
Dairy cows	2006-2007	Belgium	11/118 mastitis isolates	Vanderhaeghen et al., 2010
Dairy cows	2008	Germany	5.1 – 16.7%	Spohr et al., 2011
Chickens	Not given	Belgium	12.8% of broiler farms	Nemati et al., 2008

Pig

A study in pigs in the Netherlands by De Neeling et al. (2007) found 209 (39%) of pigs to carry MRSA in their nares, and from the same study reported that 81% of pigs farms had carrier animals. Wulf and Voss (2008) revealed that more than 20% of pig farmers and 39% of slaughterhouse pigs in the Netherlands are positive for MRSA strains ST398, and this study showed the potential of MRSA transmission between pigs and pig farmers. In Germany, Tenhagen et al. (2009) found MRSA positives in 368 (70.8%) of 520 nasal swab samples from pigs from 4 slaughterhouses, with a minimum of 58.5% positives and a maximum of 80.0% positives. A study in Ontario in Canada found MRSA colonization rates of 25% among pigs and 20% among pig farmers, and the predominant strain was ST398 (59.2%) (Khanna et al., 2008).

Poultry

A study in poultry in Belgium by Nemati et al. (2008) found 12.8% of Belgian broiler farms to be positive for MRSA (CC398). Similarly, Persoons et al. (2009) revealed a new *spa* type within CC398 to be present in 2/14 randomly selected broiler farms (14.3%), but it was not found in 10 layer farms.

2.8 Extended-spectrum beta-lactamases producing *Escherichia coli* or ESBLs-producing *E.coli*

Escherichia coli are gram negative, rod-shaved bacteria and are classified as part of the Enterobacteriaceae family. Normally, these bacteria inhabit the gastrointestinal tract of warm blooded animals. Most *E. coli* strains are harmless, but some can cause serious food borne infections in humans, and these organisms are the most important single pathogen involved in clinical cases in dairy cattle especially in herds with low bulk milk somatic cell counts.

Beta-lactamases are enzymes produced by bacteria and are responsible for their resistance to beta-lactam antibiotics like penicillins. These antibiotics have a common structure known as a beta-lactam, and lactamase enzymes make these antibiotics ineffective against beta-lactamase producing bacteria. Members of the family Enterobacteriaceae commonly express plasmid-encoded beta-lactamases, such as TEM-1, TEM-2, and SHV-1 that make these bacteria resistant to penicillins but not to extended-spectrum cephalosporins. Extended-spectrum beta-lactamases (ESBLs) are beta-lactamases that

hydrolyze extended-spectrum cephalosporins which include cefotaxime, ceftriaxone, and ceftazidime as well as the oxyimino-monobactam aztreonam. The ESBLs are frequently plasmid encoded and the plasmids frequently carry genes encoding resistance to other drug classes (for example, aminoglycosides). Therefore, antibiotic options in the treatment of ESBLs-producing organisms are extremely limited.

In human medicine, ESBLs-producing *E. coli* can cause a wide range of infections, ranging from urinary tract infection to severe blood poisoning. Infections with ESBLs-producing *E. coli* have become a particular problem in recent years, as these strains of bacteria are becoming more common, and they are highly resistant to many classes of antimicrobial agents. Infections by ESBLs-producing *E. coli* strains are difficult to treat.

2.8.1 ESBLs-producing *E. coli* in humans in Germany

In human medicine in Germany, *E. coli* is a frequent cause of urinary tract infections and it accounts for 15% of nosocomial bacteremia. In addition, *E. coli* is responsible for abdominal infections, wound infections, pneumonia, and meningitis. In hospitals for the last 10 to 15 years, in *E. coli* rising rates of resistance were recorded for many antimicrobials, notably fluoroquinolones, numerous beta-lactams, and trimethoprim-sulfamethoxazole. The increase of resistance against the groups of third and fourth generation cephalosporins (such as cefotaxime, ceftazidime, cefepime) can be explained in part by the emergence of ESBLs (extended spectrum beta-lactamase)-producing organisms (GERMAP, 2008).

2.9 Antimicrobial use in human medicine in Germany

In Germany, information on the antimicrobial consumption for the community setting is primarily derived from databases of health insurance plans (Wissenschaftliches Institut der AOK, WldO). The total consumption of antibiotics per year in human medicine in Germany can be estimated to be in the range of 250 – 300 tons, and 85% of all prescriptions are in the community setting (GERMAP, 2008). For the year 2007, total outpatient prescriptions of antibiotic was 360.2 Mio. Defined Daily Doses (DDD). Penicillins (oral penicillin or amoxicillin), tetracycline, new macrolides, oral cephalosporins, and fluoroquinolones are the most common by prescribed substances (Table 4).

The antibiotic use in outpatients can be best described in the form of DDD per 1,000 population and day(DDD/1.000). Prescription density in the outpatient area in Germany

during 2003 – 2007 was scattered between 13 and 15 DDD/1.000, and in the year 2007 was 14.8 DDD/1.000. Compared with other countries across Europe, Germany's outpatient antibiotic consumption fell into the lower third of the range. The consumption is comparable to that in neighboring countries Switzerland, Austria, the Netherlands and Denmark, but much lower than for example in Poland, Belgium, Luxembourg or France (GERMAP, 2008).

Table 4. Data on antibiotic consumption for the community setting in Germany in 2007 from databases of health insurance plans (WldO) (adapted from GERMAP, 2008)

Antibiotic classes	Prescribed daily doses (Million DDD)
Penicillins (oral penicillins or amoxicillin)	105.0
Tetracycline	84.0
New Macrolides (including azalides/ketolides)	47.1
Oral Cephalosporins	38.0
Fluoroquinolones	35.5
Sulfonamides/Trimethoprim	21.1
Erytromycin/ Old Macrolides	8.3
Nitrofurans	8.0
Clindamycin (including fusidix acid)	6.8
Aminopenicillin/ Beta-Lactamase Inhibitor and Flucloxacillin	6.4
Total	360.2

Table 5 shows data of changes in the prescription of the antibiotic classes in the outpatient setting during 2003 – 2007 in Germany. From databases of health insurance plans (WldO), there are significant increases in the prescription of oral cephalosporins (31%), fluoroquinolones (33%), and nitrofurans (33%). The prescription of penicillins (oral penicillins or amoxicillin) in community setting during 2003 – 2007 is roughly constant, while there are decreases in the prescriptions of tetracyclines (-7%), erythromycin and old macrolides (-10%), and sulfonamide/trimethoprim (-13%).

Table 5. Changes in the prescription of the antibiotic classes for the community setting in Germany in the period 2003 – 2007 from databases of health insurance plans (WldO) (adapted from GERMAP, 2008)

Antibiotic classes	Change (%)
Penicillins (oral penicillins or amoxicillin)	-4
Tetracycline	-7
New Macrolides (including azalides/ketolides)	+8
Oral Cephalosporins	+31
Fluoroquinolones	+33
Sulfonamides/Trimethoprim	-13
Erytromycin/ Old Macrolides	-10
Nitrofurans	+33
Clindamycin (including fusidix acid)	+2
Aminopenicillin/ Beta-Lactamase Inhibitor and Flucloxacillin	+12

The main data sources for hospital antimicrobial consumption came from the surveillance projects MABUSE network (Medical Antibiotic Use Surveillance and Evaluation) and SARI (Surveillance of Antibiotic consumption and Resistance in Intensive care). Antibiotic use in the residential sector is in the best form defined by daily doses (DDD) or recommended daily doses (RDD) per 100 patient days (DDD/100 or RDD/100).

Table 6. European studies on antibiotic use density in hospitals (data DDD/100 from 2004 or earlier) and comparison with situation in the USA (adapted from GERMAP, 2008)

Region (hospitals)	DDD/100
Sweden (n=80)	59
Denmark (n=66)	64
The Netherlands (n=86)	58
Germany (n=184)	50
Europa (n=139)	50
USA (n=130)	79

The estimated volume of hospital antibiotic consumption in Germany for the year 2004 is about 50 DDD per 100 patient days in acute care hospitals, and this estimated volume is similar to those reported for other countries (Table 6).

Table 7 shows data for the top 15 prescribed antimicrobial agents used in hospitals in Germany in 2004. For oral administration, the most frequently used antimicrobial substances in the hospitals are cefuroxime axetil (7.9%) and trimethoprim / sulfamethoxazole (cotrimoxazole) (7.9%). Cefuroxime (5.9%) is also the most frequently used parenteral substance.

Table 7. Data for the top 15 prescribed antimicrobial agents used in hospitals in Germany in 2004 from databases of the surveillance project MABUSE network (adapted from GERMAP, 2008)

Parenteral antibiotics	%	Oral antibiotics	%
Cefuroxime	5.9	Cefuroxime axetil	7.9
Ceftriaxone	5.5	Cotrimoxazol	7.9
Cefazolin	2.5	Ciprofloxacin	6.0
Ampicillin/Sulbactam	2.4	Amoxicillin	4.5
Piperacillin	1.7	Levofloxacin	4.4
Vancomycin	1.4	Sultamicillin	4.4
Clindamycin	1.4	Amoxicillin/Clavulanic acid	4.0
Amoxicillin/Clavulanic acid	1.4	Cefaclor	2.3
Imipenem	1.4	Clindamycin	2.0
Ciprofloxacin	1.4	Clarithromycin	2.0
Gentamicin	1.1	Roxithromycin	1.9
Piperacillin/Tazobactam	1.1	Moxifloxacin	1.7
Cefotiam	1.1	Penicillin V	1.6
Cefotaxime	1.1	Erythromycin	1.6
Levofloxacin	1.0	Doxycycline	1.5

2.10 Antimicrobial use in veterinary medicine

2.10.1 Mechanism of action of antimicrobial agents

Antimicrobial agents used for the treatment of bacterial infections can be categorized according to their principle mechanism of action into 4 groups (Tenover, 2006):

1. Interference with cell wall synthesis: Antimicrobial agents that work by inhibiting bacterial cell wall synthesis include the beta-lactams, such as penicillins, cephalosporins, carbapenems, and monobactams, and the glycopeptides, including vancomycin and teicoplanin.
2. Inhibition of protein synthesis: Bacterial ribosomes differ in structure from eukaryotic cells. Antimicrobial agents take advantage of these differences to selectively inhibit bacterial growth. Macrolides, aminoglycosides, and tetracyclines bind to the 30S ribosomal subunit. Whereas chloramphenicol binds to the 50S subunit of the ribosome.
3. Interference with nucleic acid synthesis: Fluoroquinolones exert their antibacterial effects by disrupting DNA synthesis and causing lethal double strand DNA breaks during DNA replication.
4. Inhibition of a metabolic pathway: Sulfonamides and trimethoprim block the enzymatic pathway for bacterial folate synthesis, which ultimately inhibits DNA synthesis.

2.10.2 Usage of antimicrobial agents in food producing animals

Modern food producing animal production is very intensive with optimization of every step in the production. Most food animals in industrialized countries are reared in large groups on small areas and with an attempt to achieve quick weight gains. Consequently, a large number of substances with antimicrobial activity are used in modern animal production systems.

In food animal production, antimicrobial agents are normally used in one of the four different ways (Aarestrup, 2005):

1. Therapy: Treatment of infections in clinical sick animals.
2. Metaphylactics: Treatment of clinical-healthy animals belonging to the same flock or pen as animals with clinical signs. In this way, infections may be treated before

they become clinically visible and the entire treatment period may thereby be shortened.

3. Prophylactics: Treatment of healthy animals in a period where they are stressed in order to prevent disease, for example medication at weaning, vaccination, transport, and mixing of animals. During such periods, animals are generally recognized as more susceptible to infection, and long-term experience with the animal production systems requires the application of antimicrobials at such times to avoid the onset of infections. Without these preventive treatments, subsequent clinical infections would occur more frequently and would require more therapeutic interventions for an efficient control.
4. Growth promotion: Inclusion of antimicrobials continuously in animal feed at low concentrations to improve growth during the entire growth period of animals. The growth promotion is specific to food producing animals.

2.10.3 Antimicrobial agents use in veterinary medicine in Europe

In Europe, in 1997, the total sales volumes of antibiotics was 10,493 tonnes of active ingredients. These sales volumes can be subdivided into 5,400 t for human health usage (52%), 3,494 t for animal health use (33%), and 1,599 t for growth promotion (15%) (Schwarz and Chaslus-Dancla, 2001). Differences in percentages of drugs used for therapy or growth promotion exist between the different countries (Table 8).

Table 8. Sales volumes of antimicrobial agents as therapeutics and growth promoters in different EU member states in 1997 (adapted from EMEA, 1999)

Country	Sales of growth promoters		Sales of therapeutics	
	Tonnes of active substances	% of the EU market	Tonnes of active substances	% of the EU market
Austria	23	1	8	< 1
Belgium-Luxembourg	110	7	125	4
Denmark	75	5	60	2
Finland	< 1	< 1	12	< 1
France	339	21	492	14
Germany	255	16	488	14
Greece	15	1	110	3
Ireland	34	2	22	< 1
Italy	100	6	389	11
The Netherlands	226	14	300	9
Portugal	24	2	44	1
Spain	198	12	616	18
Sweden	< 1	< 1	20	< 1
UK	191	12	788	23
Total	1,590	100	3,474	100

In 1997, tetracyclines accounted for 66% of the total sales volumes of antimicrobial agents use for therapeutical reasons in veterinary medicine in Europe and Switzerland, followed by macrolides (12%), penicillins (9%), and aminoglycosides (4%). Sales volumes of fluoroquinolone (1%) and trimethoprim/sulfonamides (2%) played a minor role for therapeutic use (Table 9).

Table 9. Sales volumes of antimicrobial agents as therapeutics in the EU and Switzerland in 1997 (adapted from EMEA, 1999)

Classes of antimicrobial	Tonnes of active substances	% of total
Tetracycline	2,294	66
Macrolides	424	12
Penicillins	322	9
Aminoglycosides	154	4
Trimethoprim/sulfonamides	75	2
Fluoroquinolones	43	1
Other classes	182	5
Total	3494	100

2.10.4 Antimicrobial agents use in veterinary medicine in Germany

Antibiotics have almost exclusively activity against bacteria, and any use of antibiotics in veterinary medicine can lead to development of resistance. Use of antimicrobial agents is thought to be essential for the treatment and health maintenance of animals. In Germany, guidelines for the use of antibiotics in animals are provided by the Bundestierärztekammer (BTK) or Germany's National Veterinary Association. The guidelines are valid for every application of antibiotics in "good veterinary practice ". They are therefore not only for the treatment of bacterial diseases of farm animals, but also for the treatment of individual, small and pet animals. Antibiotics should only be used to treat sick animals against bacterial infections, and used under veterinary prescription. The use of antibiotics always requires a diagnosis based on appropriate clinical and laboratory examination, and the appropriate antibiotic is selected due to criteria, including spectrum activity, antimicrobial resistance, therapeutic range, type of effect, and pharmacokinetics (Bundestierärztekammer, 2010).

Antibiotics are used for treatment, prevention and control of bacterial diseases. In veterinary medicine, the use of antibiotics is considered for animal welfare and determined by the livestock side, mainly by the objective of use of animals to produce healthy food. The information of antimicrobial consumption in veterinary medicine in Germany is monitored by the Panel of the Veterinary Association for Consumer Research (GfK).

In Germany, the nationwide sales figures of antimicrobial agents for use in veterinary medicine increased by 9% to a total of 784 t during the years 2003 to 2005

(Table 10). The use of tetracyclines showed a significant change, in the year 2005 35 t were used less than in 2003. The changes is correlated with expired licenses of several low-price oral tetracyclines in Europe in this period. A significant increase of 44 t was found in the use of beta-lactams, and this increases is correlated especially with the falling price of amoxicillin.

Between 2003 to 2005, the number of pigs increased by more than 1 million. The moderate increase in antibiotic use is explained by the increase in production and by changing the mode of production and good value of active ingredients. The total production in the poultry and cattle sectors remained roughly constant during the investigation period.

Table 10. Use of veterinary antibiotics in Germany in the year 2003 and 2005. Data from the Panel of the Veterinary Association for Consumer Research (GfK) (adapted from GERMAP, 2008)

Group of antibiotic	2003	2005
	Tonnes (%)	Tonnes (%)
Aminoglycosides	27.3 (3.8)	36.3 (4.6)
Beta-lactams	155.2 (21.4)	199.2 (25.4)
Quinolone	3.5 (0.5)	3.7 (0.5)
Lincosamide	7.5 (1.0)	12.1 (1.5)
Macrolides	38.6 (5.3)	52.6 (6.7)
Phenicol	4.7 (0.6)	4.8 (0.6)
Pleuromutilin	6.8 (0.9)	6.4 (0.8)
Polypeptide	23.4 (3.2)	21.8 (2.8)
Sulfonamide	71.7 (9.9)	97.5 (12.4)
Tetracycline	385.5 (53.2)	350.0 (44.6)
Total	724.2 (100)	784.4 (100)

In 2005, tetracyclines accounted for almost 45 % of all antimicrobial agents in veterinary use, followed by beta-lactams (25.4 %), sulfonamides (including trimethoprim) (12.4%), and macrolides (6.7%). Consumption of fluoroquinolones (3.7 tonnes), chloramphenicol (4.8 tonnes) and pleuromutilin (6.4 tons) remained at low levels and played only a minor role in all antimicrobial agents in veterinary use (GERMAP, 2008).

2.11 The WHO list of critically important antimicrobials

The WHO list of critically important antimicrobials was based on the following criteria for categorization as developed by two Expert Meetings (WHO, 2005; WHO, 2007):

- **Criterion 1** Sole therapy or one of few alternatives to treat serious human disease.
- **Criterion 2** Antibacterial use to treat diseases caused by organisms that may be transmitted via non-human sources or diseases causes by organisms that may acquire resistance genes from non-human sources.

The definitions of the different categories were as follows:

Critically important antimicrobials are those that meet criteria 1 and 2

Highly important antimicrobials are those that meet criteria 1 or 2

Important antimicrobials are those that meet neither criteria 1 nor 2

Table 11 shows the categorization of antimicrobials used in human medicine according to their importance in the treatment of disease.

Table 11. Categorization of antimicrobials used in human medicine according to importance in the treatment of disease (adapted from FAO/WHO/OIE, 2008)

Critically important antimicrobials	Highly important antimicrobials	Important antimicrobials
Aminoglycosides	Amidinopenicillins	Cyclic polypeptides
Ansamycin	Aminoglycosides	Fosfomycin
Carbapenems	Amphenicols	Fusidic acid
Cephalosporins (3^{rd} and 4^{th} generation)	Cephalosporins (1^{st} and 2^{nd} generation)	Lincosamides
Glycopeptides	Cephamycins	Mupirocin
Macrolides	Clofazimine	Nitrofurantoins
Penicillins (natural, aminopenicillin and antipseudomonal)	Monobactams	Nitroimidazoles
Quinolones	Penicillins (antistaphylococcal)	
Streptogramins	Polymyxins	
Tetracyclines	Sulfonamides	
Drugs used solely to treat tuberculosis or other mycobacterial diseases		

2.12 The OIE list of critically important antimicrobials

Following a recommendation from the 2nd Joint FAO/WHO/OIE Expert Meeting in Oslo 2004, the OIE initiated the process of developing a list of critically important antimicrobials in veterinary medicine. The fundamental aim of this list is to safeguard the efficacy and availability of veterinary antimicrobial products for animal diseases where there are few or no alternatives. Additionally, the following utilities were expected:

- To help veterinarians in their choice of the appropriate therapeutic agent.
- To complement the OIE guidelines for responsible and prudent use of antimicrobial agents.
- To serve as a useful information base to support science-based risk assessment of antimicrobial resistance.

The critically important antimicrobials in veterinary medicine were defined as

"... antimicrobials used for the treatment, prevention and control of serious animal infections that may have important consequences on animal health and welfare, public health or important economic consequences and where there are few or no alternatives."

2.12.1 Development of the OIE list

The work was assigned to the OIE *ad hoc* Group on Antimicrobial Resistance, consisting of experts, who report to the OIE Biological Standard Commission. The OIE list was developed on the basis of replies to a questionnaire sent to all 167 OIE member countries and to four international organizations that have signed a cooperation agreement with OIE. This methodology was chosen to reflect, to the extent possible, the real use and need of antimicrobials under various conditions among OIE member countries worldwide.

In this questionnaire the following four basic topics were explored:

- Animal species.
- Disease treated and causative microbe: Seriousness and economic importance.
- Antimicrobials used: Type of use (treatment/prevention/control), route, accessibility of the product, and quality of the substance.
- Specific rules of usage for the country concerned.

2.12.2 Criteria used for categorization of veterinary antimicrobials

The following criteria were selected to determine the degree of importance for classes of veterinary antimicrobials.

Criterion 1 Response rate to the questionnaire regarding Veterinary Critically Important Antimicrobials.

This criterion was met when a majority of the respondents (more than 50%) identified the importance of the antimicrobial class in their response to the questionnaire.

Criterion 2 Treatment of serious animal disease and availability of alternative antimicrobials.

This criterion was met when compounds within the class were identified as essential against specific infections and there was a lack of sufficient therapeutic alternatives.

On the basis of these criteria, the following three categories were established:

• Veterinary *critically important* antimicrobials are those that meet criteria 1 and 2

• Veterinary *highly important* antimicrobials are those that meet criteria 1 or 2

• Veterinary *important* antimicrobials are those that meet neither criteria 1 nor 2.

Table 12 shows the categorization of antimicrobials used in veterinary medicine according to their importance in treatment of disease.

Table 12. Categorization of antimicrobials used in veterinary medicine according to their importance in treatment of disease (adapted from FAO/WHO/OIE, 2008)

Veterinary critically important antimicrobials	Veterinary highly important antimicrobials	Veterinary important antimicrobials
Aminoglycosides	Rifamycins	Bicyclomycin
Cephalosporins	Fosfomycin	Fusidic acid
Macrolides	Ionophores	Novobiocin
Penicillins	Lincosamides	Orthosomycins
Phenicols	Pleuromutilins	Quinoxalines
Quinolones	Polypeptides	Streptogramins
Sulfonamides		
Tetracycline		

OIE has ranked veterinary antimicrobial agents as critically important, highly important or important to animal health, and WHO has ranked human antimicrobial agents as critically important, highly important or important to human medicine; most classes of antimicrobial agents have been ranked by both OIE and WHO in their lists. The comparison of the human and veterinary lists developed by WHO and OIE, respectively, shows that most antimicrobial classes are used in both human and in veterinary medicine. When just the critically important antimicrobials are examined, some classes appear on the WHO list (carbapenems, ansamycins, glycopeptides, streptogramins and oxazolidinones), whereas other classes appear only on the OIE list (phenicols, sulfonamides, diaminopyrimidines and tetracyclines). For a number of classes there is an overlap, where the class of antimicrobial agents is listed as critically important for human health by WHO and as critically important for animal health by OIE. These are 3rd and 4th generation cephalosporins, quinolones (including fluoroquinolones), macrolides, penicillins and aminoglycosides. This overlap highlights the need to have in place antimicrobial resistance surveillance and to identify and implement appropriate management measures in order to mitigate resistance dissemination and maintain the efficacy of the drugs. Prudent use of all antimicrobials is considered essential.

Recommendations to FAO, WHO, OIE and national governments were developed that address the risk analysis process of hazards related to antimicrobial resistance resulting from the use of antimicrobials in food animals. Both lists of critically important antimicrobials should be revised on a regular basis (e.g. every second year) in a collaborative and coordinated approach by FAO, OIE and WHO. When revising the lists of critically important antimicrobials, specific consideration should be given to a harmonized classification of cephalosporins, macrolides, aminoglycoside and tetracyclines, if possible to the compound level, taking into account that the resistance mechanism may be different for each generation of antimicrobials. With respect to the OIE list of critically important antimicrobials, it is suggested to further refine the categorization of critically important drugs with respect to their importance in the treatment of specific animal disease.

Antimicrobial resistance monitoring of foodborne pathogens and commensals (animal, human, food and commodity) should be implemented by all countries considering risk management measures, to enable the detection of hazards and to accurately assess the success of selected interventions. Ideally, quantitative standardized minimum inhibitory concentration methods should be applied. Foodborne pathogens and commensals (in

particular *Salmonella* spp., *Campylobacter* spp. and *Escherichia coli*) linked to potential antimicrobial resistance to 3rd and 4th generation cephalosporins, quinolones and macrolides should be given special consideration for risk analysis.

The regulatory process should encompass elements that emphasize improvements in animal husbandry that lead to a better animal health status and, consequently, decreases the need for antimicrobial use. When antimicrobial drugs are used, prudent use of these drugs should be promoted, particularly in respect of WHO and OIE identified critically important antimicrobials. Surveillance data for animals, humans and food are an integral part of ensuring correct regulatory policies and their effect in preventing and/or containing antimicrobial resistance. Table 13 shows the categorization of veterinary antimicrobials for cattle.

Table 13. Categorization of veterinary antimicrobials for cattle (FAO/WHO/OIE, 2008)

Antimicrobial family	VCIA	VHIA	VIA	Specific comments
Aminoglycosides Aminocyclitol Spectinomycin Aminoglycosides Streptomycin Dihydrostreptomycin Framycetin Kanamycin Neomycin Apramycin Gentamicin	Y			The wide range of applications and the nature of the diseases treated make aminoglycosides extremely important for veterinary medicine. Aminoglycosides are of importance in septicaemias, digestive, respiratory and urinary diseases.
Ansamycin-Rifamycins Rifaximin		Y		This antimicrobial class is authorized only in a few countries and with a very limited number of indications (mastitis).
Bicyclomycin Bicozamycin			Y	Biclomycin is listed for digestive and respiratory diseases in cattle.
Cephalosporins Cephalosporins 1st generation Cefacetrile Cefalexin	Y			Cephalosporins are used in the treatment of septicaemias, respiratory infections and mastitis.

Antimicrobial family	VCIA	VHIA	VIA	Specific comments
Cefapyrin Cefazolin Cefalonium Cephalosporins 2nd generation Cefuroxime Cephalosporins 3rd generation Cefoperazone Ceftiofur Ceftriaxone Cephalosporins 4th generation Cefquinome				
Fosfomycin Fosfomycin		Y		This antimicrobial is authorized only in a few countries.
Fusidic acid Fusidic acid			Y	Fusidic acid is used in the treatment of ophthalmic diseases in cattle.
Ionophores Lasalocid Monensin		Y		**Ionophores are used only in animals** Ionophores are essential for animal health because they are used to control intestinal parasitic coccidiosis (*Eimeria* spp.) where there are few or no alternatives available.
Lincosamides Pirlimycin Lincomycin		Y		
Macrolides Azalide Tulathromycin Macrolides C14 Erythromycin Macrolides C16 Spiramycin Tilmicosin Tylosin	Y			Macrolides are used to treat liver abscesses (*Fusobacterium necrophorum*) and respiratory infections in cattle.
Novobiocin Novobiocin			Y	**Novobiocin is only used in animals** Novobiocin is used in the treatment of mastitis in form of intramammary creams.

Antimicrobial family	VCIA	VHIA	VIA	Specific comments
Penicillins Natural Penicillins Benzylpenicillin Penethamate hydroxide Penicillin procaine Amdinopenicillins Mecillinam Aminopenicillins Amoxicillin Ampicillin Hetacillin Aminopenicillins plus beta-lactamase inhibitor Amoxicillin_Clavulanic Carboxypenicillins Ticarcillin Tobicillin Ureido penicillin Aspoxicillin Antistaphylococcal penicillins Cloxacillin Dicloxacillin Nafcillin Oxacillin	Y			Penicillins are used in the treatment of septicaemias, respiratory and urinary tract infections. They are very important in the treatment of many diseases in a broad range of animal species.
Phenicols Florphenicol Thiamphenicol	Y			Phenicols represent a useful alternative in respiratory infections of cattle, and in particular florfenicol, are used to treat pasteurellosis in cattle.
Polypeptides Bacitracin Polypeptides cyclic Colistin Polymixin		Y		Polypeptides are indicated in septicaemias, colibacillosis, salmonellosis and urinary infections. Cyclic polypeptides are widely used against Gramnegative digestive infections.
Quinolones Quinolones 1st generation Flumequin Nalidixic acid	Y			Quinolones of the 1st and of 2nd generations are used in septicaemias and in infections such as colibacillosis, which cause serious losses in cattle and other species.

Antimicrobial family	VCIA	VHIA	VIA	Specific comments
Oxolinic acid Quinolones 2nd generation (Fluoroquinolones) Ciprofloxacin Danofloxacin Difloxacin Enrofloxacin Marbofloxacin Norfloxacin Orbifloxacin				
Sulfonamides Sulfadiazine Sulfadimerazin Sulfadimethoxine Sulfadimidine Sulfadimethoxazole Sulfanilamide Sulfaquinoxaline Sulfonamides+ Diaminopyrimidines Sulfamethoxypyridazine Trimethoprim+Sulfonamide Diaminopyrimidines Trimethoprim	Y			Several sulfonamides alone or in combination with diaminopyramidines are very essential because of diseases covered (bacterial, coccidial and protozoal infections), and used in multiple animal species.
Streptogramins Virginiamycin			Y	Virginiamycin is an important antimicrobial in the prevention of necrotic enteritis.
Tetracyclines Tetracycline Chlortetracycline Oxytetracycline Doxycycline	Y			Tetracyclines are very important in the treatment of many bacterial and chlamydial diseases in a broad range of animal species. There are no alternatives to tetracyclines in the treatment of animals against heartwater (*Ehrlichia ruminantium*) and anaplasmosis (*Anaplasma marginale*).

VCIA = Veterinary Critically Important Antimicrobials; VHIA = Veterinary Highly Important Antimicrobials; VIA = Veterinary Important Antimicrobials

2.13 Antimicrobial usage in dairy cattle

2.13.1 Judicious use of antimicrobials for dairy cattle veterinarians

Any use of antimicrobials in human and veterinary medicine can lead to development of resistant organisms. In the United States, the veterinary profession shares the concerns of the public, governmental agencies, and of the public health community regarding the broad issue of antimicrobial resistance and specifically the potential risk of resistance developing in animals with subsequent transfer to humans. The American Veterinary Medical Association (AVMA) and the American Association of Bovine Practitioners (AABP) are committed to judicious and prudent use of antimicrobials by veterinarians for the prevention, control, and treatment of animal diseases.

The AVMA started a profession-wide initiative, including companion and food animal practitioner groups, to develop and implement judicious use principles for the therapeutic use of antimicrobials by veterinarians. The AVMA Executive Board approved a general set of judicious use principles in November 1998 (AVMA, 1998).

Judicious use of antimicrobials is an integral part of good veterinary practice. It is an attitude to maximize therapeutic efficacy and minimize selection of resistant microorganisms. Judicious use principles are a guide for optimal use of antimicrobials. They should not be interpreted so restrictively as to replace the professional judgment of practitioners or to compromise animal health or welfare. In all cases, animals should receive prompt and effective treatment as deemed necessary by the prescribing or supervising veterinarian.

The fifteen general principles are:

1. Preventive strategies, such as appropriate husbandry and hygiene, routine health monitoring, and immunizations, should be emphasized.
2. Other therapeutic options should be considered prior to antimicrobial therapy.
3. Judicious use of antimicrobials, when under the direction of a veterinarian, should meet all the requirements of a valid veterinarian-client-patient relationship.
4. Prescription, Veterinary Feed Directive, and extralabel use of antimicrobials must meet all the requirements of a valid veterinarian-client-patient relationship.
5. Extralabel antimicrobial therapy must be prescribed only in accordance with the Animal Medicinal Drug Use Clarification Act (AMDUCA) amendments to the Food, Drug, and Cosmetic Act and its regulations.

6. Veterinarians should work with those responsible for the care of animals to use antimicrobials judiciously regardless of the distribution system through which the antimicrobial was obtained.
7. Regimens for therapeutic antimicrobial use should be optimized using current pharmacological information and principles.
8. Antimicrobials considered important in treating refractory infections in human or veterinary medicine should be used in animals only after careful review and reasonable justification. Consider using other antimicrobials for initial therapy.
9. Use narrow spectrum antimicrobials whenever appropriate.
10. Utilize culture and susceptibility results to aid in the selection of antimicrobials when clinically relevant.
11. Therapeutic antimicrobial use should be confined to appropriate clinical indications. Inappropriate uses such as for uncomplicated viral infections should be avoided.
12. Therapeutic exposure to antimicrobials should be minimized by treating only for as long as needed for the desired clinical response.
13. Limit therapeutic antimicrobial treatment to ill or at risk animals, treating the fewest animals indicated.
14. Minimize environmental contamination with antimicrobials whenever possible.
15. Accurate records of treatment and outcome should be used to evaluate therapeutic regimens.

2.13.2 American Association of Bovine Practitioners prudent drug usage guidelines for cattle.

Concurrent with the AVMA initiative, the AABP was addressing antimicrobial use in cattle through articles in the Bovine Practitioner, presentations at annual meetings, and the AABP Board of Directors approved Prudent Drug Use Guidelines in March 1999 (AVMA, 2000). Following are general guidelines for the prudent therapeutic use of antimicrobials in beef and dairy cattle:

1. The veterinarian should accept responsibility for helping clients design management, immunization, housing, and nutritional programs that will reduce the incidence of disease and the need for antimicrobials.
2. The use of antimicrobials only within the confines of a valid veterinarian-client-patient relationship, for both dispensing and the issuance of prescriptions, has been

recommended by the American Association of Bovine Practitioners. In addition, extralabel usage should be within the provisions contained within the AMDUCA regulations.

3. Veterinarians should properly select and use antimicrobial drugs. Veterinarians should participate in continuing education programs that include therapeutics and emergence and/or development of antimicrobial resistance.
    - A dairy cattle veterinarian should have strong clinical evidence of the identity of the pathogen causing the disease, based upon clinical signs, history, necropsy examination, laboratory data and past experience before making a recommendation for antimicrobial use.
    - Antimicrobials should be used at a dosage and duration appropriate for the condition treated.
    - Product choices and regimens should be based on available laboratory and label (including package insert) information, additional data in the literature and consideration of the pharmacokinetics, spectrum, and pharmacodynamics of the drug.
    - Antimicrobials should be used with specific clinical outcome(s) in mind, such as fever reduction, return of mastitic milk to normal, to eliminate or reduce shedding, contagion, and recurrence of disease.
    - Periodically monitor herd pathogen susceptibility and therapeutic response, especially for routine therapy such as dry cow intramammary antibiotics, to detect changes in microbial susceptibility and to evaluate antimicrobial selections.
    - Use products that have the narrowest spectrum of activity and known efficacy in vivo against the pathogen causing the disease problem.
    - Antimicrobials should be used at a dosage appropriate for the condition treated and for as short a period of time as reasonable. Therapy should be discontinued when it is apparent that the immune system can manage the disease, reduce pathogen shedding and minimize recurrence of clinical disease or development of the carrier state.
    - When possible, antimicrobials of lesser importance in human medicine should be chosen before choosing a newer generation animal antimicrobial that may be in the same class as a human antimicrobial that may be used as the primary or sole

treatment for a human infection. An antimicrobial for which emergence of resistance is expected to be in an advanced stage, should also not be chosen.

- Antimicrobials labeled for use for treating the condition diagnosed should be used whenever possible.
- Combination antimicrobial therapy should be discouraged unless there is information to show increase in efficacy or suppression of resistance development for the target organism. Compounding of antimicrobial formulations should be avoided.
- When appropriate, local therapy (e.g. intramammary, intrauterine, topical) is preferred over systemic therapy.
- Treatment of chronic cases or those with a poor chance of recovery should be avoided.
- Prophylactic or metaphylactic use of antimicrobials should be based on a group, source or production unit evaluation rather than being utilized as standard practice.

4. Veterinarians should endeavor to ensure proper on-farm drug use. Drug integrity should be protected through proper handling, storage and observation of the expiration date.
    - Prescription or dispensed drug quantities should be appropriate to the production-unit size and expected need so that stockpiling of antimicrobials on the farm is avoided.
    - The veterinarian should train farm personnel who use antimicrobials on indications, dosages, withdrawal times, route of administration, injection site precautions, storage, handling, record keeping, and accurate diagnosis of common diseases.
    - Veterinarians are encouraged to provide written, updated protocols for diagnosis and treatment to clients whenever possible. Those protocols should describe conditions and provide instructions for antimicrobial use at a farm or unit when a veterinarian is unavailable.

2.13.3 Antimicrobial class use in dairy cattle

The antimicrobial class is defined as a group of antimicrobial agents with related molecular structures, often with a similar mode of action because of interaction with a similar target

and thus subject to similar mechanism of resistance. Variations in the properties of antimicrobials within a class often arise as a result of the presence of different molecular substitutions, which confer various intrinsic activities or various patterns of pharmacokinetic and pharmacodynamic properties (FAO/WHO/OIE, 2008).

Antimicrobial drugs are used in dairy cattle as therapeutics, growth promotion, and prophylactics. The most commonly used antimicrobial drugs in dairy cattle are usually from one of five major classes (McAllister et al., 2001; White and McDermott, 2001):

- beta-lactams (penicillin, ampicilin, cephapirin, ceftiofur, amoxicillin, and cloxacillin).
- macrolides (erythromycin).
- tetracyclines (oxytetracycline, tetracycline, and chlortetracycline).
- aminoglycosides (streptomycin, neomycin, and gentamycin).
- sulfonamides (sulfamethazine, and sulfadimethoxine).

Antimicrobial drugs treatment of dairy cattle for diseases caused by bacterial infection is a common and necessary occurrence. Antimicrobial drugs are administered to dairy cattle through intramuscular injection, intravenous injection, subcutaneous injection, orally, topically, intramammary infusion, or intrauterine infusion. In dairy cattle operation, antimicrobial drugs are administered for both therapeutic and prophylactic purposes. The major purpose of antimicrobial drugs usage in adult dairy cattle in dairy farms is therapeutically for treatment of clinically and subclinically diseases caused by bacterial infections. Some antimicrobial drugs are used for preventing diseases in healthy cattle during periods of increasing susceptibility as prophylactic purpose, such as dry cow therapy at the end of lactation in dairy cow (Aarestrup, 2005). Diseases or health problems requiring the most extensive use of antimicrobial drugs for treatment and prophylaxis in adult dairy cows are mastitis, respiratory disease, lameness, and metritis (Zwald et al., 2004; Sawant et al., 2005; Pol and Ruegg, 2007).

2.13.4 Antimicrobial drugs used for treatment of lameness, respiratory infections, and metritis in adult cows

Table 14 shows the list of antimicrobial drugs used for selected diseases, including lameness, respiratory infections, and metritis of cows. Ceftiofur is the most frequently used

antimicrobial agent for treatment of lameness (65%), respiratory infections (85%), and metritis (85%) in cows in 20 conventional dairy farms in Wisconsin (Pol and Ruegg, 2007).

Table 14. List of antimicrobial drugs used for selected diseases of cows

Diseases/health problems	Pol and Ruegg, 2007 Agent (%)	Zwald et al., 2004 Agent (%)	Sawant et al., 2005 Agent
Lameness	Ceftiofur (65) Tetracycline (55) Penicillin (25) Ampicillin (5)	Ceftiofur (58.6) Penicillin (42.4) Tetracycline (24.2) Ampicillin (4) Sulfonamide (4)	Ceftiofur Amoxicillin Oxytetracycline Florfenical Penicillin Sulfadimethoxine
Respiratory infections	Ceftiofur (85) Ampicillin (40) Tetracycline (30) Penicillin (20) Sulfonamide (20)	Ceftiofur (80.8) Penicillin (32.3) Tetracycline (31.3) Ampicillin (22.2) Sulfonamide (20.2) Florfenical (7.1) Tilmicosin (3)	Amoxicillin Ampicillin Ceftiofur Oxytetracycline Florfenical Penicillin G Sulfadimethoxine
Metritis	Ceftiofur (85) Tetracycline (60) Penicillin (35) Ampicillin (25) Sulfonamide (5)	Penicillin (43.4) Ceftiofur (41.4) Tetracycline (15.2) Ampicillin (12.1)	Ceftiofur Penicillin G

The study of antimicrobial usage on 99 conventional dairy farms in Minnesota, Michigan, Wisconsin, and New York (Zwald et al., 2004) found that farmers used antimicrobial drugs to treat adult cows with lameness (83%), respiratory infections (97%), and metritis (80%). For lameness, ceftiofur (58.6%) and penicillin (42.4%) were commonly used. Ceftiofur (80%) was the most frequently used antimicrobial agent to treat respiratory infections, and penicillin (43.4%) and ceftiofur (41.4%) were commonly used for treatment of metritis.

2.13.5 Antimicrobial drugs used for treatment of mastitis

Bovine mastitis is the single most common cause for antimicrobial drugs used in lactating dairy cattle worldwide (Grave et al., 1999; Sawant et al., 2005; Pol and Ruegg, 2007; Thomson et al., 2008). Eliminating mastitis pathogens from the dairy cows and herd is the main objective of antimicrobial treatment.

Table 15. List of antimicrobial drugs used for treatment of clinical mastitis

Treatment	Pol and Ruegg, 2007 Agent(%)	Sawant et al., 2005 Agent
Intramammary	Cephapirin (90) Pirlimycin (75) Amoxicillin (40) Cloxacillin (10) Erythromycin (10)	Amoxicillin Cephapirin sodium Cloxacillin sodium Erythromycin Hetacillin Novobiocin Penicillin G Penicillin G and Novobiocin Pirlimycin
Parenteral	Penicillin (35) Tetracycline (35) Ceftiofur (30) Ampicillin (30) Sulfonamide (20)	

Pol and Ruegg (2007) reported that cephapirin (90%) and pirlimycin (75%) were the most frequently used intramammary antimicrobial drugs for treatment of clinical mastitis in 20 conventional farms in Wisconsin, and the majority of the farmers used one or more parenteral antimicrobial drugs, including penicillin, tetracycline, ceftiofur, ampicillin, and sulfonamide, to treat about one-third of clinical mastitis cases (Table 15).

A survey study on antibiotic usage in dairy herds in Pennsylvania, Sawant et al. (2005) found that clinical mastitis was observed on all farms (N = 33) and was reported to be the most common condition for antimicrobial treatment in lactating cows. Intramammary antimicrobial drugs were used to treat clinical mastitis in 14% of lactating cows and cephapirin sodium (49%) was a preferred drug for the farmers in Pennsylvania. Intramammary antimicrobial drugs including penicillin G, penicillin G and novobiocin, amoxicillin, cloxacillin, cephapirin, erythromycin, hetacillin, novobiocin and pirlimycin are approved for use in lactating dairy cattle in the US (Sawant et al., 2005).

In the study of antimicrobial drug usage in 20 conventional dairy farms in Wisconsin Pol and Ruegg (2007) reported that intramammary antimicrobial treatments were used in all quarters of all cows on all conventional farms at dry-off. Most commonly used for intramammary dry cow therapy were penicillin (90%), streptomycin (90%), and cephapirin (75%).

Table 16. List of antimicrobial drugs used for dry cow therapy

Treatment	Pol and Ruegg, 2007 Agent(%)	Sawant et al., 2005 Agent
Intramammary	Penicillin (90)	Benzathine Cephapirin
	Streptomycin (90)	Benzathine Cloxacillin
	Cephapirin (75)	Erythromycin
	Novobiocin (15)	Novobiocin
		Penicillin G
		Penicillin G and Novobiocin
		PenicillinG and Streptomycin
Parenteral	Penicillin (25)	
	Tetracycline (20)	
	Tylosin (20)	

In addition, the same study found that nine conventional dairy farms regularly used parenteral antimicrobial drugs, including penicillin, tetracycline, and tylosin, at dry-off (Table 16). In the dairy herds in Pennsylvania, benzathine cephapirin (52%) was most frequently used for dry cow therapy for the farmers and benzathine cephapirin, benzathine cloxacillin, erythromycin, novobiocin, penicillin G, penicillin G and novobiocin, and penicillin G and streptomycin are intramammary antimicrobial drugs approved for dry cow therapy (Sawant et al., 2005).

Antimicrobial drugs use has been suggested as a selective force in determining the bacterial ecology of bovine mastitis, and any use of antimicrobial agents will to some extent benefit the development of resistance strains. As in human medicine, the use of antimicrobial agents in food producing animals creates a selective pressure for the emergence and dissemination of antimicrobial-resistant bacteria, including animal

pathogens, human pathogens that have food animal reservoirs, and commensal bacteria that are present in food animals. These resistant bacteria may be transferred to humans either through the food supply or by direct contact with animals. Moon et al. (2007) indicated that antimicrobial treatment of infectious diseases in animals poses the risk of selection of resistant strains and introduction of these strains into the food chain. Antimicrobial resistance is an important public health concern worldwide. Therefore, antimicrobial usage in dairy cattle should be applied restrictedly.

2.13.6 Antimicrobial susceptibility of *S. aureus* and *E. coli* isolated from dairy cattle

Staphylococcus aureus is one of the major contagious mastitis pathogens and is a common cause of mastitis in dairy cows. Antimicrobial therapy is one of the most important elements for controlling *S. aureus* mastitis. Antimicrobial susceptibility tests assist the veterinarian in selecting the most appropriate antimicrobial agent for treatment of intramammary infection caused by *S. aureus*. Therefore, the most extensive antimicrobial resistance studies in dairy cattle have been investigated in *S. aureus* isolated from the milk of dairy cows with mastitis or from submission to diagnostic laboratories.

Table 17. Minimum inhibitory concentrations (MIC) of antimicrobial agents against *Staphylococcus aureus* isolated from dairy cows in Germany

Antimicrobial agent	De Oliveira et al. (2000)		Tenhagen et al. (2006)	
	MIC_{90}	Range	MIC_{90}	Range
Ampicillin	2.0	≤0.06 to 64.0	2.0	0.03 to 32.0
Oxacillin	1.0	≤0.125 to >64.0	0.5	0.06 to 128.0
Amoxicillin+clavulanic acid	≤0.06	≤0.06	1.0	0.125 to 1.0
Cephalothin	0.5	≤0.06 to >64.0		
Ceftiofur	1.0	0.25 to >64.0		
Cefquinome			1.0	0.008 to 16.0
Gentamycin			0.5	0.125 to 64.0
Streptomycin			16.0	0.5 to 256.0
Neomycin	2.0	0.06 to 16.0		
Erythromycin	0.5	0.125 to >64.0		

The antimicrobial susceptibility study (disk diffusion method) of mastitis pathogens by Erskine et al. (2002) reported that ampicillin (50.4%) and penicillin (50.4%) are the antimicrobial drugs to which *S. aureus* are most commonly resistant. The results from the same study demonstrated that *S. aureus* isolated were susceptible to cephalothin (99.8%), ceftiofur (99.8%), erythromycin (93.1%), gentamicin (98.9%), oxacillin (99.4%), pirlimycin (97.9%), sulfa-trimethoprim (99.4%), and tetracycline (91.5%).

From the study of antimicrobial resistance in dairy cows in Germany by Tenhagen et al. (2006), minimum inhibitory concentrations (MIC) were determined for 199 strains of *S. aureus* isolated from clinical healthy udder quarters of dairy cows. The antimicrobial agents tested were ampicillin, oxacillin+2%NaCl, cefquinome, amoxicillin+clavulanic acid, gentamicin, and streptomycin. The MIC_{90} for these antimicrobial agents were 2.0, 0.5, 1.0, 1.0, 0.5, and16.0 µg/ml, respectively (Table 17). De Oliveira et al. (2000) reported the MIC for 811 strains of *S. aureus* isolated from bovine mastitis in 11 countries, and found only small variations between countries. For 103 strains of *S. aureus* isolated from bovine mastitis in Germany, MIC_{90} for ampicillin, oxacillin, amoxicillin+clavulanic acid, cephalothin, ceftiofur, neomycin, and erythromycin were 2.0, 1.0, ≤ 0.06, 0.5, 1.0, 2.0, and 0.5 µg/ml, respectively.

Environmental mastitis pathogens are commonly found in the cow resting environment and *Escherichia coli* is one of the most important environmental mastitis pathogens. Erskine et al. (2002) reported antimicrobial susceptibility from the disk diffusion method of *E. coli* isolated from bovine mastitis cases in the United States for ampicillin, cephalothin, ceftiofur, gentamicin, sulfa-trimethoprim, and tetracycline to be 84.3, 74.5, 95.4, 98.0, 97.2, and 66.8 %, respectively.

2.14 Bovine mastitis

Bovine mastitis is defined as the inflammation of the mammary gland that can have an infectious and non-infectious etiology. The majority of mastitis cases are due to an intramammary infection (IMI) caused by microorganisms. Different microorganisms, including bacteria, mycoplasma, yeast, and algae have been reported to cause IMI (Wilson et al., 1997; Bradley, 2002; Bradley et al., 2007), from which several bacterial species are the most common cause. Basically, bovine mastitis is divided into two main classes. The first is clinical mastitis which manifests visible abnormalities in the milk or the udder, or

both. The other is subclinical mastitis which produces no visible signs from udder or milk except when using diagnostic tools.

Bacterial mastitis pathogens are typically classified as either contagious or environmental, based upon their primary reservoir and mode of transmission. Contagious mastitis pathogens can be considered as organisms adapted to survive in the mammary gland of the cow. The major contagious mastitis pathogens include *Streptococcus agalactiae*, *Staphylococcus aureus*, *Mycoplasma spp.*, and *Corynebacterium bovis*. They are capable of establishing subclinical mastitis, which manifests as an elevation in the somatic cell count of milk from the affected quarter. The primary reservoir of contagious mastitis pathogens is the udder of the infected cows. Milk from infected quarters or infected cows is the main source of bacterial pathogens for uninfected quarters or uninfected cows. They typically spread from quarter to quarter or from cow to cow around the time of milking. The use of dry cow therapy and post milking teat and milking cluster disinfection are important control procedures for most contagious mastitis pathogens.

Environmental mastitis pathogens are described as opportunistic invaders of the mammary gland. They are not adapted to survive within the mammary gland, and are commonly found in the cows resting environment including soil, feces, and bedding. The primary reservoir of these pathogens is the environment in which the cows are living and not the infected quarter in the herd. The exposure of the teat end to the environmental mastitis pathogens is not limited to only the milking time. It can occur during milking, between milking, and during the dry period. Programs that successfully control contagious mastitis pathogens do not effectively control mastitis caused by environmental pathogens. Control of mastitis caused by the environmental pathogens depends on decreasing the exposure of the teat ends to environmental mastitis pathogens and by increasing the resistance of the cows to IMI. The major environmental mastitis pathogens comprise coliform bacteria (*Escherichia coli* and *Klebsiella spp*.) and environmental streptococci, species of streptococci others than *Streptococcus agalactiae* (e.g. *Streptococcus uberis*).

2.14.1 The distribution of mastitis pathogens

The distribution of mastitis pathogens may differ between clinical and subclinical mastitis, as clinical mastitis can be caused by pathogens that are present for a short period and produce obvious clinical signs. Conversely, subclinical mastitis can be caused by pathogens that are present for longer periods and produce no visible clinical signs.

From a survey study of mastitis pathogen on dairy farms in England and Wales Bradley et al. (2007) found that *Streptococcus uberis* (23.5%), *E. coli* (19.8%), and Coagulase-negative staphylococci (CNS, 8.1%) were the pathogens most frequently isolated from cases of clinical mastitis (Table 18), and CNS (14.9%), *Streptococcus uberis* (13.8%), and *S. aureus* (5.2%) were the most prevalent mastitis pathogens isolated from the cases of subclinical mastitis (Table 19). In cases of clinical mastitis, the most frequently isolated pathogens from a university dairy herd in the U.S. reported by Roberson et al. (2004), were *Streptococcus uberis* (29.1%), *E. coli* (19.4%), and CNS (4.9%), and the study by Tenhagen et al. (2008) in primiparous and older cows in dairy herds in Germany, streptococci were the predominant finding (32.7 and 39.2%), followed by CNS (27.4 and 16.4%) and coliforms (10.3 and 13.1%).

Table 18. Distribution of mastitis pathogens from clinical mastitis cases (percent)

Pathogens	Bradley et al., 2007	Roberson et al., 2004	Tenhagen et al., 2008	
			Cows	Heifers
Staphylococcus aureus	3.3	2.9	11.7	10.0
Streptococcus uberis	23.5	29.1	-	-
Streptococci	-	-	39.2	32.1
Escherichia coli	19.8	19.4	-	-
Coliforms	-	-	13.1	10.3
CNS	8.1	4.9	16.4	27.4
No growth	26.5	11.7	19.5	21.3

In subclinical mastitis cases, the most prevalent mastitis pathogens in dairy herds in Germany (Tenhagen et al., 2006) were CNS (9.1%), *S. aureus* (5.7%), and *Streptococcus uberis* (1.0%). Ferguson et al. (2007) reported that CNS (14.6%), *S. aureus* (12.5%), and *Streptococcus uberis* (6.6%) were the most frequently isolated mastitis pathogens in dairy herds in Ragusa, Sicily (Table 19).

Table 19. Distribution of mastitis pathogens from subclinical mastitis cases (percent)

Pathogens	Bradley et al., 2007	Ferguson et al., 2007	Tenhagen et al., 2006
Staphylococcus aureus	5.2	12.5	5.7
Streptococcus uberis	13.8	6.6	1.0
Escherichia coli	3.0	-	-
Coliforms	-	1.7	0.3
CNS	14.9	14.6	9.1
No growth	38.6	64.6	73.6

2.14.2 The importance of bovine mastitis

Mastitis still remains the most costly disease in the dairy industry worldwide. The economic losses are due to decreased milk production, increased replacement cost, discarded milk, treatment cost, extra labor cost, and negative effects on milk quality (Seegers et al., 2003). In a study on clinical mastitis cases, the average estimated cost was 179$ because of milk yield losses, increased mortality, and treatment cost (Bar et al., 2008). In the U.S. dairy herds, an average loss associated with subclinical mastitis was 110$ per cow, mostly due to reduced milk production (Ott, 1999). Caraviello et al. (2005) reported that the risk of culling for Holstein cows with lactation average somatic cell count (SCC) more than 700,000 cells per ml was 3.4, 2.7, or 2.3 times greater, respectively, than that of Holstein cows with SCC of 200,00o to 250,000 cells per ml in herds with low, medium, and high average SCC. In dairy heifers, elevated SCC may negatively effect milk production during the first lactation (De Vliegher et al., 2005). Prepartum antibiotic treatment of heifers will reduce the prevalence of IMI after treatment (Owens et al., 2001) and is effective to reduce the rate of clinical mastitis in heifers during lactation (Oliver et al., 2003). Subclinical and clinical mastitis, especially during the first 90 days of lactation, have been implicated in decreasing reproductive performance, including increased days in milk at first service, increased services per conception, and increased days not pregnant (Schrick et al., 2001; Ahmadzadeh et al., 2008). The importance of bovine mastitis is not only the economic losses in the dairy industry but also the importance of mastitis in public health. Most dairy farms require the use of antimicrobial drugs for treatment of sick dairy cattle, and mastitis is the most common disease of lactating dairy cattle to be treated with antimicrobial drugs (Grave et al.,

1999; Zwald et al., 2004; Swant et al., 2005; Pol and Ruegg, 2007; Thomson et al., 2008). The extensive use of antimicrobial drugs for treatment and control of mastitis has possible implications for human health through an increasing risk of antimicrobial residues in milk products (McEwen et al., 1991; Ruegg and Tabone, 2000; Ruegg, 2005). Moreover, antimicrobial drugs usage may exert selective pressure for antimicrobial resistant strains of bacteria that may enter the food chain (Schwarz and Chaslus-Dancla, 2001; White and McDermott, 2001; Lee, 2003; Aarestrup, 2005). The food safety issues, the spread of zoonotic organisms and antimicrobial resistant strains of bacteria via milk and milk products are of greater concern especially in the countries that produce raw milk cheese and have a niche market for unpasteurised dairy products.

2.14.3 Risk factors associated with clinical mastitis

Several studies have investigated risk factors for clinical mastitis in dairy herds. Different results have been reported, and some risk factors are in accordance. Risk factors associated with clinical mastitis in dairy herds can be divided into three groups of factors, including resistance of the cow to intramammary infection, exposure to mastitis pathogens, and environment and management. Breed of the cows, milk production, milk somatic cell count and nutrition are risk factors for clinical mastitis, which have influenced resistance of the cow to IMI. Schukken et al. (1990) reported that breed was a risk factor for clinical mastitis in dairy herds in the Netherlands. The increased rate of clinical mastitis in the dual purpose Meusse-Rhine-IJssel (MRIJ) breed versus the Holstein-Friesians may be associated with udder conformation or a genetic trait. Similarly, in a study in Sweden, it was found that a low incidence rate of veterinary-treated clinical cases of mastitis was associated with having a herd of the Swedish Red & White or Swedish Red Breed (SRB) (Nyman et al., 2007).

An increase in the mean milk yield of the herd is associated with an increased risk for clinical mastitis (Schukken et al., 1990; Waage et al., 1998; O'Reilly et al., 2006). In the study on low somatic cell score herds in France Barnouin et al. (2005) reported that herds with 305-d milk yield of more than 7,435 kg had a higher incidence rate of clinical mastitis, and in the Netherlands Barkema et al. (1999) found the milk production of cows in low bulk milk somatic cell count herds to be positively associated with the incidence rate of clinical mastitis caused by *E. coli*.

A decrease in the bulk milk somatic cell count is associated with an increase in the incidence rate of clinical mastitis in the herd (Waage et al., 1998; Suriyasathaporn et al., 2000). Milk somatic cells are primarily leukocytes, which include macrophages, lymphocytes, and polymorphonuclear leukocytes. A higher number of somatic cells would indicate more leukocytes in milk and these may be able to kill microorganisms by themselves or to initiate an inflammatory response better than a low number of somatic cells (Suriyasathaporn et al., 2000).

Leaking milk outside the parlor is found to be an important risk factor for mastitis (Schukken et al., 1990; Peeler et al., 2000; O'Reilly et al., 2006). The teat canal is the first line of defense mechanism to protect the cow from intramammary infections by microorganisms. Leaking milk between milking means a continuous opening of the teat orifice, which is associated with an increased risk of mastitis pathogens penetrating the teat canal and colonizing the gland. Vitamin E and selenium are essential micronutrients and have a direct effect on the function of the immune system. Deficiencies in either of these micronutrients have been related to increased incidences and severity of clinical mastitis (Smith et al., 1984; Hogan et al., 1993; Smith et al., 1997; Weiss, 2002).

Factors that reduced the exposure to mastitis pathogens include lowering animal density (Barkema et al., 1999), separating parlor for diseased cows (Barkema et al., 1999), using a cloth to dry the teats after washing (O'Reilly et al., 2006), offering feed after milking (Peeler et al., 2000), and a pasture rotation policy of grazing dry cows for a maximum of 2 weeks before allowing the pasture to remain nongrazed for a period of 4 wk (Green et al., 2007). These factors are associated with a decrease in the incidence of clinical mastitis in the dairy herds. Factors associated with an increase in the incidence of clinical mastitis are dirty cubicles (Schukken et al., 1990), cows with very dirty udders (Schukken et al., 1990; Nyman et al., 2007; Breen et al., 2009), less frequent cleaning out the straw yard (O'Reilly et al., 2006), and udder preparation with water (Schukken et al., 1991).

Management and environmental factors, including increasing detection of mastitis by stripping foremilk before attaching the clusters (Peeler et al., 2000; O'Reilly et al., 2006) and calving in late spring or summer (Waage et al., 1998) are associated with an increase in the incidence of clinical mastitis. Routine body condition scoring of cows at drying off (Green et al., 2007), selection of dry cow treatments for individual cows (Whist et al., 2006; Green et al., 2007), and culling chronically infected cows (Barnouin et al., 2005; Whist et al., 2006) are farm management factors associated with a reduced rate of clinical mastitis.

2.15 *Staphylococcus aureus* mastitis

Staphylococcus aureus is one of the most common causes of mastitis on dairy farms and is regarded as a major contagious mastitis pathogen. Mastitis caused by *S. aureus* can result in both clinical and subclinical mastitis. *S. aureus* can adapt to survive in the mammary gland of the cow, and IMI by this organism may be present for a long period and cause an elevation of somatic cell count in affected quarters. Mastitis caused by *S. aureus* in dairy herd can be assessed by monitoring somatic cell count of milk from individual cow or of bulk tank milk (Smith et al., 1985; Green et al., 2004). Infected quarters of lactating dairy cows are the major source of *S. aureus* in a dairy herd, and the transmission typically occurs from cow to cow during milking time. Contagious mastitis caused by *S. aureus* can effectively be controlled by implementation of a mastitis control program including rapid identification and treatment of clinical mastitis cases, whole herd antibiotic dry cow therapy, post milking teat disinfection, culling of chronically infected cows, and routine maintenance of milking machines (Natzke, 1981; Dodd, 1983; Smith, 1983; Oliver and Mitchell, 1984; Harmon, 1996). A decrease in bulk tank milk somatic cell count is an indicator of the success of the mastitis control program. Herds with low bulk milk somatic cell counts have been able to decrease the prevalence of mastitis with contagious pathogens (Wilson et al., 1995; Suriyasathaporn et al., 2000).

Despite a great deal of progress in mastitis control programs, the IMI caused by *S. aureus* still remain in dairy herds (Olde Riekerink et al., 2006; Ferguson et al., 2007). The study on contagious mastitis pathogens in bulk tank milk of 258 dairy farms in Prince Edward Island reported that *S. aureus* was isolated in bulk tank milk from 191 (74%) dairy farms (Olde Riekerink et al., 2006). Ferguson et al. (2007) reported that prevalence of *S. aureus* in Ragusa, Sicily, was 12.5% of milk samples, 20.6% of cows, and 88.1% of herds. The prevalence of *S. aureus* mastitis is varied, depending on the studies, herds, and countries. Prevalence of *S. aureus* isolated from cases of clinical mastitis has been reported to range from 2.9 to 11.7% of infected quarters (Roberson et al., 2004; Bradley et al., 2007; Olde Riekerink et al., 2008; Tenhagen et al., 2008) and from 5.2 to 12.5% of quarter samples in subclinical mastitis cases (Tenhagen et al., 2006; Breadley et al., 2007; Ferguson et al., 2007).

2.15.1 Transmission of *Staphylococcus aureus* infections

The major source of *Staphylococcus aureus* is the infected mammary gland of lactating cows, but these bacteria have been found on the teat skin, teat lesions, muzzles, nostrils, and from body sites (Matos et al., 1991; Roberson et al., 1994; Roberson et al., 1998). The most common transmission pathway is bacteria from an infected quarters spreading to uninfected quarters by contaminated teat cup liners, milker's hands, and common udder cloths or sponges. The milking period and milking parlor represent the time period and the place where most new *S. aureus* IMI occur. When milking, if the last cow milked has *S. aureus* IMI, then the next cow milked with the same unit will be directly exposed to these bacteria from the residue milk in the teat cup liners. Used common udder cloths can be a major means of spreading *S. aureus*, as nearly every cow in the herd would be exposed.

Other important sources of *S.aureus* are infected heifers and *S. aureus* colonized teat skin (Trinidad et al., 1990; Matos et al., 1991; Roberson et al., 1994; Roberson et al., 1998). Generally, dairy heifers are assumed to have good udder health, and have been thought of as a group of uninfected. Consequently, mastitis control programs are not emphasized on dairy heifers, and their mammary glands and secretions are not closely observed until calving. However, IMI in unbred and prepartum dairy heifers have been frequently reported (Trinidad et al., 1990; Oliver et al., 1992; Nickerson et al., 1995; Aarestrup and Jensen, 1997; Owens et al., 2001; Oliver et al., 2003; Borm et al., 2006; Compton et al., 2007; Fox, 2009), and many studies have reported a high incidence of clinical mastitis and a high prevalence of IMI in first-calving heifers (Pankey et al., 1991; Barkema et al., 1998; Compton et al., 2007; Sampimon et al., 2009). The pathogens that cause mastitis in dairy heifers are the same as those that cause infections in the multiparous dairy cows in the herds. Moreover, many studies indicated that IMI in a quarter before parturition increases the risk of IMI after parturition (Oliver and Sordillo, 1988; Aarestrup and Jensen, 1997; Roberson et al., 1998). In dairy heifers, IMI caused by *S. aureus* prepartum and at first parturition have been reported (Trinidad et al., 1990; Pankey et al., 1991; Nickerson et al., 1995). Trinidad et al. (1990) found 37.1% of 116 unbred and primigravid dairy heifers in four herds to be positive for *S. aureus*, while in the study of Pankey et al. (1991) only 2.6% of 382 heifers within 3 d postpartum on 11 dairy farms were diagnosed. *S. aureus* do not persist on healthy skin but readily colonize damaged skin and teat lesions. Milking machine irritation, chemical irritation, frostbite, and fly bites greatly increase the probability for developing *S. aureus* infection. The organisms multiply in the lesions and result in an

increased chance of teat canal colonization and subsequent udder infection. Roberson et al. (1994) reported that 35% of 700 heifers were colonized with *S. aureus* on a body site and heifers with teat skin colonized by *S. aureus* were 3.34 times more likely to have *S. aureus* IMI at parturition than non-colonized heifers. Heifers with persistently colonized body sites represent the primary reservoir of *S. aureus* for other heifers, and contact among them may be an important mode of transmission of *S. aureus* leading to IMI in heifers at parturition (Roberson et al., 1998). In addition, Aarestrup and Jensen (1997) reported a large increase in the number of IMI caused by *S. aureus* shortly after parturition, and indicated that heifers shortly after parturition are exposed to this bacterial species and are at great risk of acquiring an intramammary infection.

2.15.2 Control of *Staphylococcus aureus* mastitis

Staphylococcus aureus IMI may be present for a long period in the udder and can persist through the lactation and into subsequent lactations. To control mastitis caused by *S. aureus* is very difficult. Effective control programs should be based on prevention of new infections and elimination of infected cows by antibiotics treatment or by culling.

Limiting the spread of *S. aureus* organisms from cow to cow is necessary for prevention of new IMI caused by *S. aureus* in the herd. *S. aureus* infected cows should be identified and milked last (Hutton et al., 1990) or with a separate milking unit (Wilson et al., 1995). Milking hygiene practice and an effective post-milking teat dip should be applied (Dodd, 1983; Harmon, 1996). Antibiotic treatments in both lactating and dry cows are an important part of a *S. aureus* control program for eliminating or reducing infected cows in the herds (Barkema et al., 2006). Cows that are subclinically infected with *S. aureus* are a primary and important source of infection for other lactating cows in the herd, and clinical mastitis sometimes occurs following subclinical infections. Antibiotic treatment during lactation is aimed to eliminate infections and may improve the clinical condition. The cure rate for *S. aureus* mastitis treatment during lactation has been reported to range from 15.4 to 33.6 % (Sol et al., 1997; Bradley and Green, 2009). Because of the relatively low probability of cure, it is important to know risk factors for cure. The most important factors associated with cure are cow, pathogen, and treatment (Sol et al., 1997; Barkema et al., 2006). Treatment effectiveness is decreased in older cows with high SCC and with lactation progress (Sol et al., 1997). Treatment of young animals with penicillin-sensitive *S. aureus* infections is recommended, whereas treatment of older animals with chronic infections or

penicillin-resistant *S. aureus* infections should be discouraged (Barkema et al., 2006). Dry cow therapy is more effective to eliminate *S. aureus* IMI than lactating treatment. Cure rates for *S. aureus* mastitis treatment at drying off range from 65.8 to 78.1% of quarters infected (Sol et al., 1994; Nickerson et al., 1999; Dingwell et al., 2003). Culling of chronically infected cows is an important part of control programs for *S. aureus* mastitis in dairy herds, by reducing the number of *S. aureus* infected cows in a herd. Chronically infected cows with *S. aureus*, which have not responded to antibiotic treatment, should be considered for culling.

First calving heifers represent a valuable current and future resource. They make up the largest parity group in most herds, usually have the highest genetic merit of any age group in the herd, and, until a calf or milk is sold following their first calving, have not generated any revenues. Intramammary infections caused by *S. aureus* are commonly found in heifers, either prepartum or during early lactation (Trinidad et al., 1990; Pankey et al., 1991; Oliver et al., 1992; Nickerson et al., 1995), and these infections can become clinical and increase the chance of spread to other cows in the herd. Pregnant heifers should not be housed together with dry cows, when a significant number of cows in the herd are known to be infected with *S. aureus*. If heifers are purchased, segregate them until milk samples can be cultured and their mastitis pathogen status can be determined. Several management practices can be used on heifers prior to calving to eliminate or reduce infection before parturition. Administration of dry cow therapy to heifers at 60 days before expected calving date (Nickerson et al., 1995) or treating heifers with lactating cow antibiotic treatment 14 days before the expected calving date (Oliver et al., 1992) have been effective to reduce prevalence of *S. aureus* IMI at calving, and this practice is recommended in herds with significant heifer mastitis caused by *S. aureus*.

2.16 *Escherichia coli* mastitis

Many countries have implemented mastitis control programs and have been able to successfully decrease the prevalence of mastitis with contagious pathogens. A decrease in bulk tank milk SCC is an indicator of the success of mastitis control programs. However, at the same time period there has been an increase in the incidence of mastitis caused by environmental pathogens, including coliform bacteria and environmental streptococci. *Escherichia coli*, *Klebsiella spp.*, and *Enterobacter spp.* are common gram negative bacteria that cause mastitis in dairy cows. These organisms could be classified under the simple term

of coliform mastitis. *Escherichia coli* is a normal inhabitant of the gastrointestinal tract of warm blooded animals, and this organism is the most important single pathogen involved in clinical cases of mastitis, especially in herds with low bulk milk SCC. Programs that successfully control contagious mastitis do not control clinical mastitis caused by environmental pathogens.

Many studies have reported the prevalence of *E. coli* or coliform bacteria IMI in both clinical and subclinical cases. The percentage of *E. coli* or coliforms isolated for clinical mastitis cases ranges from 8.4 to 42.8% (Smith et al., 1985; Suriyasathaporn et al., 2000; Roberson et al., 2004; Kalmus et al., 2006; Bradley et al., 2007; Olde Reikerink et al., 2008; Tenhagen et al., 2008) and the prevalence of *E. coli* or coliforms IMI in subclinical mastitis ranges from 0.3 to 3.0% of samples (Tenhagen et al., 2006; Bradley et al., 2007; Ferguson et al., 2007). In the study on low bulk milk SCC farms Suriyasathaporn et al. (2000) found the predominant mastitis pathogens isolated to be *E. coli* (42.8% of samples from 969 cases of clinical mastitis). Moreover, in a study on clinical mastitis in freshly calved heifers, Kalmus et al. (2006) reported that the most frequently isolated pathogen was *E. coli* (22.1%). Factors associated with an increased rate of *E. coli* or coliform mastitis in dairy herds are low bulk milk SCC herds (Schukken et al., 1989; Barkema et al., 1998; Suriyasathaporn et al., 2000), summer season (Smith et al., 1985; Hogan and Smith, 2003; Olde Riekerink et al., 2007), total confinement housing (Olde Riekerink et al., 2007), housing on sawdust bedding (Zdanowicz et al., 2004), management practices such as low frequency of cubicle cleaning, use of rubber mats in the calving area, and udder preparation with water (Schukken et al., 1991). The cow factors include early lactation period, increased parity, dry period especially during the early and late dry period (Smith et al., 1985; Hogan and Smith, 2003).

2.16.1 Control of *Escherichia coli* mastitis

Control of environmental mastitis, including *E. coli* mastitis, can be achieved by decreased exposure of teat ends to environmental pathogens and by increased resistance of cows to infections (Smith et al., 1985).

Decreased exposure of teat ends to environmental pathogens by reducing pathogens in the environment of dairy cows will not be easy. Organic bedding materials, such as sawdust and recycle manure, have the ability to support the growth of environmental pathogens. In summer, high temperature and humidity in total confined herds can enhance

the growth of *E. coli*, resulting in high bacterial counts in bedding (Smith et al., 1985; Hogan et al., 1989). Bacterial counts in bedding materials are correlated with bacterial counts on the teat ends (Zdanowicz et al., 2004). Increased teat ends exposure to mastitis pathogens in bedding is associated with a high incidence of clinical mastitis (Hogan et al., 1989; Olde Riekerink et al., 2007). The recommended bedding material for controlling environmental mastitis is washed sand, which has little nutritive value to support the growth of mastitis pathogens (Hogan and Smith, 1998). The study of free-stall mattress bedding treatments to reduce mastitis bacterial growth by Kristula et al. (2008) found that hydrated lime was a treatment that significantly reduced *E. coli* and *Klebsiella spp.* counts on both mattress and teat ends. Management such as udder preparation with water and rubber mats in the calving area (Schukken et al., 1991) and housing management, including low animal density and keeping the cows on pasture in summer (Barkema et al., 1999) are factor associated with exposure of teat ends to environmental pathogens. Moreover, internal sealant used with dry cow intramammary antibiotics for dry cow therapy has been reported for preventing new IMI during dry and early lactation periods (Godden et al., 2003; Newton et al., 2008).

Increasing the resistance of the cows to infections is one of the important parts of a mastitis control programs for environmental pathogens. Management in the dry and early lactation periods, such as providing a clean, dry environment, and good ventilation, are important for the prevention of mastitis in dry and calving cows (Green et al., 2007). Supplementation of vitamin E and selenium has been reported to have a positive effect on udder health as it can reduce the incidence and duration of clinical mastitis (Smith et al., 1984; Hogan et al., 1993; Weiss, 2002).

Chapter 3
Materials and Methods

3.1 Materials

3.1.1 Dairy Farms

A total of 60 dairy farms located in the regions of Brandenburg (n= 18), Lower Saxony and Saxony Anhalt (n= 42) in Germany were included in this study. All farms were selected by convenience sampling and visited once between February and September 2010 by veterinarians of the Diagnostic Service of the Clinic for Cattle and Swine, Faculty of Veterinary Medicine, Freie Universität Berlin, the Animal Health Service of Saxony Anhalt or the Department of Microbiology, University of Applied Sciences and Arts, Hannover.

3.1.2 Questionnaire

A form was developed, which included questions on the following topics: farm characteristics, health management in dairy cattle, treatment strategies emphasizing the usage of antimicrobials. In detail, the 55 questions addressed milk quota, herd size and composition (7 questions), replacement heifer management (4 questions), contact with other animal species (2 questions), occurrence of diseases and health problems as well as antimicrobial usage in cows (12 questions), occurrence of diseases and health problems as well as antimicrobial usage in calves (12 questions), decision criteria to send milk samples for bacteriological examination (6 questions), decision criteria to treat a cow with mastitis (3 questions), treatment and management in case of clinical mastitis (6 questions), and dry cow treatment and management (3 questions). The questions were answered by the herd manager while the form was filled in by the veterinarian during a routine farm visit. It took approximately 30 min to complete the form.

3.1.3 MRSA isolates

A total of 36 MRSA isolates from bovine milk were included in this study. Five isolates were obtained from the MRSA positive strains of the 60 dairy farms in this study, and 31 isolates originated from the Bundesinstitut für Risikobewertung (BfR; Federal Institute for Risk Assessment), including 13 isolates from a national monitoring project on bulk tank

milk (ZoMo 2009), and 18 other bulk tank milk isolates submitted to the National Reference Laboratory (NRL) in 2009 and 2010 in the framework of other projects.

3.1.4 Sample collection from bulk tank milk

At the farm visit the veterinarian obtained milk samples from the bulk tank, which were chilled immediately after sampling and subsequently transported to the laboratory. At the laboratory, all milk samples were kept refrigerated and processed within 24 to 72 h.

3.2 Methods

3.2.1 Bacterial isolation and identification

3.2.1.1 Extended-spectrum beta-lactamases producing *Escherichia coli* (ESBLs-producing *E. coli*)

For ESBLs-producing *E. coli*, 25 ml of the bulk tank milk sample was enriched by adding Lauryl Sulfate (LS) broth (Merck, Darmstadt, Germany) containing 8 µg/ml vancomycin (Sigma-Aldrich Chemie, Munich, Germany) at a 1:10 dilution to the sample and incubated at 37ºC for 20 to 24 h. One loop (10 µl) of the enriched sample was streaked onto MacConkey (MAC) agar (Merck, Darmstadt, Germany) supplemented with cefotaxime (Sigma-Aldrich Chemie, Munich, Germany) at 1 µg/ml (CTX1) and incubated at 37ºC for 20 to 24 h. Lactose-positive and bile salt precipitated-positive colony from CTX1 was streaked onto MAC agar supplemented with cefotaxime at 2 µg/ml(CTX2) and incubated at 37ºC for 20 to 24 h. Presumptive ESBLs-producing *E. coli* isolate, lactose-positive and bile salt precipitated-positive colonies from CTX2, were selected. Identification of the presumptive *E. coli* isolate was confirmed by using an API 20E kit (Biomérieux, Craponne, France). The isolate of confirmed ESBLs-producing *E. coli* was suspended in Brain Heart Infusion (BHI) broth (Oxoid, Wesel, Germany) and incubated at 37ºC for 20 to 24 h. The suspension (0.9 ml) was added to 0.9 ml Luria-Bertani (LB) broth (Merck, Darmstadt, Germany) with 80% glycerol solution and the mixture was frozen at -80ºC and -20ºC for later identification and characterization.

3.2.1.2 Methicillin-resistant *Staphylococcus aureus* (MRSA)

For bacterial isolation of MRSA, a volume of 25 ml from the bulk tank milk sample was added to 225 ml of Mueller Hinton broth (MHB) (Oxoid, Wesel, Germany) with 6.5%

NaCl and incubated aerobically at 37ºC for 16 to 20 h. 1 ml of the latter broth was then inoculated into 9 ml Tryptone Soy Broth (TSB) (Merck, Darmstadt, Germany) containing 3.5 mg/L cefoxitin (Sigma-Aldrich Chemie, Munich, Germany) and 75 mg/L aztreonam (Sigma-Aldrich Chemie, Munich, Germany) and incubated for a further 16 to 20 h at 37ºC (EFSA, 2007). One loop-full of this suspension was spread onto a chromogenic agar selective for MRSA (Brilliance MRSA Agar, Oxoid, Wesel, Germany) and incubated for 24 h at 37ºC. Based on colony morphology and colour, colonies indicative for being MRSA were subcultivated on sheep blood agar plates (BAP). After 24 h incubation at 37ºC the BAP were read. One colony that had specific colony morphology with or without hemolysis was suspended in BHI broth and incubated at 37ºC for 20 to 24 h. The suspension was tested for coagulase by tube coagulation test. The suspension (0.9 ml) of the presumptive MRSA was added to 0.9 ml LB broth with 80% glycerol. The mixture was frozen at -80ºC and -20ºC for later identification and characterization.

3.2.2 Antimicrobial susceptibility testing

For 18 strains of ESBLs-producing *E. coli*, antimicrobial susceptibility testing was performed by the agar disk diffusion method as recommended by the Clinical and Laboratory Standard Institute (CLSI, 2009a). For this purpose, Mueller Hinton agar (MHA) (Oxoid, Wesel, Germany) was used and cells were harvested from the surface of the medium with a cotton swab after 24 h growth at 37ºC. Cells were suspended in sterile saline solution (0.85% NaCl), cells density was adjusted to a 0.5 McFarland turbidity standard, and the diluted cells were plated. *E. coli* ATCC 25922 was used as the quality control strain. The following panel of 15 antimicrobial agents (Oxoid) for enterobacteria were tested: ampicillin (AMP; 10 µg), chloramphenicol (CHL; 30 µg), florfenicol (FLO; 30 µg), gentamicin (GEN; 10 µg), kanamycin (KAN; 30 µg), ciprofloxacin (CIP; 5 µg), nalidixic acid (NAL; 30 µg), amoxicillin with clavulanic acid (AMC; 20 and 10 µg), tetracycline (TET; 30 µg), streptomycin (S; 10 µg), spectinomycin (SPE; 100 µg), sulfonamide (SU; 300 µg), trimethoprim (TMP; 5 µg), sulfametoxazole and trimethoprim (SXT; 23.75 and 1.25 µg), and ceftiofur (EFT; 30 µg). In addition, all strains of ESBLs-producing *E. coli* were tested against 15 beta-lactam antmicrobial agents: ampicillin (AMP; 10 µg), cephalothin (CEF; 30 µg), cefuroxime (CXM; 30 µg), ticarcillin (TIC; 75 µg), piperacillin (PIP; 100 µg), ceftiofur (EFT; 30 µg), ceftriaxone (CRO; 30 µg), imipenem (IMP; 10 µg), cefotaxime (CTX; 30 µg), amoxicillin with clavulanic acid (AMC; 20 and 10 µg), ceftazidime (CAZ;

30 µg), aztreonam (AZM; 30 µg), cefpodoxime (CPD; 10 µg), cefoxitin (FOX; 30 µg), and cefepime (FEP; 30 µg). Evaluation of antimicrobial resistance was based on performance standards for antimicrobial susceptibility testing as recommended by the Clinical and Laboratory Standard Institute (CLSI, 2009b).

For the MRSA strains from milk samples, antimicrobial susceptibility was based on the minimum inhibitory concentration (MIC) determined by the broth micro dilution method in accordance with instruction of the Clinical Laboratory Standard Institute (CLSI, 2008). In short, custom-made microtitre plate panels were used (TREK Diagnostic Systems, Magellan Biosciences, West Sussex, England). Mueller Hinton agar (MHA) plates were incubated for 24 h at 37ºC and then used for analyses. Colonies were suspended in 5 ml of sterile saline solution (0.85% NaCl), and cell density was adjusted to a 0.5 McFarland turbidity standard. Subsequently, 30 µl of this suspension was added to Mueller Hinton broth (11 ml supplemented with Mg^{2+} and Ca^{2+}) and automatically filled into the microtiter plate. After 18 - 24 h incubation at 37ºC the plates were read. The MIC level was defined as the minimum concentration of antimicrobial agent that inhibited visible growth. The MIC of 13 antimicrobial agents/combinations of agents (concentration range in mg/L) were tested, including chloramphenicol (2 – 256), ciprofloxacin (0.5 – 64), clindamycin (0.25 – 32), erythromycin (0.125 – 16), gentamicin(0.5 – 64), kanamycin (8 – 128), linezolid (1 – 16), mupirocin (1 – 16), oxacillin (0.5 – 8), quinupristin-dalfopristin (0.5 – 8), trimethoprim-sulfamethoxazole (0.25/4.8 – 16/304), tetracycline (1 – 64), and vancomycin (2 – 32). *S. aureus* ATCC 25923 was used as the quality control strain. Evaluation of resistance was based on epidemiological cut-off values published by the European Committee for Antimicrobial Susceptibility Testing for MRSA and *S. aureus* (www.eucast.org).

3.2.3 MRSA: DNA extraction for multiplex PCR, SCC*mec* typing, and *spa* typing

Chromosomal DNA was extracted using the RTP Spin Bacteria DNA Mini Kit (Invitek, Berlin, Germany). Briefly, according to the manufacturer's instruction, isolates of confirmed MRSA strains from frozen stocks were streaked onto blood agar and incubated at 37ºC for 20 to 24 h. Isolates from blood agar were suspended in BHI broth and incubated at 37ºC for 20 to 24 h. 1 ml of the suspension was transferred into a 1.5 ml tube, centrifuged for 6 min at 13,000 rpm and the supernatant was discarded. The remaining pellet was subsequently resuspended in 400 µl resuspension buffer. The bacteria were lysed by transfering the resuspension sample into the extraction tube and vortexing it shortly. The

sample was incubated in a thermomixer for 10 min at 37ºC, at 65ºC for 10 min and at 95ºC for 5 min. 400 µl of binding buffer was added to the sample and vortexed shortly. The sample was transferred into the RTA spin filter set and incubated for 1 min at room temperature. Subsequently the sample was centrifuged at 12,000 rpm for 1 min, and the filtrate was discarded. 500 µl of wash buffer I was added to the sample and centrifuged at 10,000 rpm for 1 min, and the filtrate was discarded. 700 µl of wash buffer II was added to the sample and centrifuged at 10,000 rpm for 1 min. The filtrate was discarded and centrifuged for 3 min at 13,000 rpm to remove the ethanol from the wash buffer completely. The RTA spin filter was placed into a new 1.5 ml receiver tube, and 200 µl of elution buffer was added. The sample was incubated for 1 min at room temperature and centrifuged for 1 min at 8,000 rpm. DNA extraction in 1.5 ml receiver tube was stored at -20ºC until use for confirmation of MRSA by multiplex PCR and for characterization of SCC*mec* types, and *spa* typing.

3.2.4 Confirmation of MRSA by multiplex PCR

All 36 MRSA isolates were confirmed by multiplex PCR (Poulsen et al., 2003). According to the multiplex PCR protocol of Poulsen et al. (2003), with slight modifications, this method included simultaneous detection of the 16S rDNA specific for *Staphylococcus* species, the *S. aureus*- specific region of the thermonuclease gene (*nuc*), and the resistance gene *mec*A. PCR was carried out in volumes of 25 µl using 2.5 µl of chromosomal DNA diluted 1:10 as template. Amplifications were carried out in GeneAmp 2720 Thermal cycle (Applied Biosystems). The thermal cycle run condition was: denaturation step at 94ºC for five minutes follow by 30 cycles of 94ºC for 45 seconds, 55ºC for 45 seconds, 72ºC for 60 seconds, and a final elongation step at 72ºC for five minutes. The PCR amplified products were separated by electrophoresis at 100V for 40 min through 2.0% agarose gel and visualized under UV light.

3.2.5 SCC*mec* typing

All MRSA isolates were SCC*mec* typed using a modified protocol for the multiplex PCR described by Zhang et al. (2005). This multiplex PCR identifies SCC*mec* types I to V. PCR was carried out in volumes of 25 µl using 2.5 µl of chromosomal DNA diluted 1:10 as template. Amplifications were carried out in GeneAmp 2720 Thermal cycle, and the following run condition was used: initial denaturation step at 94ºC for five minutes followed

by 30 cycles of 94ºC for 30 seconds, 55ºC for 30 seconds, 72ºC for 45 seconds, and final extension step at 72ºC for five minutes. The PCR amplification products were visualized using a UV light box after electrophoresis at 100V for 40 min through a 2% agarose gel.

3.2.6 *spa* typing

All isolates were characterized by *spa* typing (Shopsin et al., 1999) and PCR was carried out in volumes of 50 µl using 5 µl of chromosomal DNA diluted 1:10 as template. Amplifications were carried out in GeneAmp 2720 Thermal cycle, and the following run condition was used: initial denaturation step at 94ºC for five minutes followed by 30 cycles of 94ºC for 30 seconds, 60ºC for 30 seconds, 72ºC for 30 seconds, and final extension step at 72ºC for five minutes. The PCR amplification products were visualized using a UV light box after electrophoresis at 100V for 40 min through a 2% agarose gel. Sequencing of PCR products was performed by QIAgen (Hilden, Germany). The *spa*-types were determined using Ridom Staph Type software (Ridom GmbH, Würzburg, Germany).

3.2.7 MRSA: DNA microarray analysis

Genotyping of staphylococcal DNA was tested using array hybridization kit for DNA-based detection of resistance genes and pathogenicity markers of *Staphylococcus aureus* and assignment of unknown *S. aureus* isolates to known strains based on the Staphy Type™ Kit (Clondiag, Jena, Germany). This kit detected the presence of genes encoding for antimicrobial resistance, including for methicillin, oxacillin (*mec*A), Beta-lactams (*mec*A, *bla*I, *bla*R, *blaZ*), marcolides (*erm*A, *erm*B, *erm*C, *msr*A, *mef*A, *mpb*BM), lincosamides (*erm*A, *erm*B, *erm*C, *lin*A, *cfr*), streptogramin (*erm*A, *erm*B, *erm*C, *vat*A, *vat*B, *vga*, *vga*A, *vgb*), aminoglycoside (*aac*A-*aph*D, *aad*D, *aph*A), streptothricin (*sat*), trimethoprim (*dfr*A), fusidic acid (*far*, Q6GD50), mupirocin (*mup*R), tetracycline (*tet*K, *tet*M, *tet*Efflux), chloramphenicol (*cat*, *fex*A), and vancomycin (*van*A, *van*B, *van*Z).

Moreover, the presence of genes encoding staphylococcal enterotoxins (*ent*A, *ent*A-320E, *ent*A-N315, *ent*B, *ent*C, *ent*CM14, *ent*D, *ent*E, *ent*G, *ent*H, *ent*I, *ent*J, *ent*K, *ent*L, *ent*M, *ent*N, *ent*N_1, *ent*O, *ent*Q, *ent*R, *ent*U, *egc*-cluster) and virulence factors, such as toxic shock syndrome toxins (*tst*1, *tst*-RF122), Pantone-Valentine Luekocidin (PVL), leukocidins (*luk*M/*luk*F-P83, *luk*D, *luk*E, *luk*X, *luk*Y-var1, *luk*Y-var2), hemolysin alpha (*hla*), hemolysins beta (*hlb*, un-truncated *hlb*), hemolysins gamma (*luk*F, *luk*S,*luk*S-ST22+ST45, *hlg*A), and hemolysin delta (*hld*) were detected.

3.2.7.1 Preparation of genomic DNA for microarray analysis

All 36 MRSA strains from frozen stocks were streaked onto blood agar and incubated at 37ºC for 20 to 24 h. Cells were lysed using lysis enhancer and lysis buffer from a Staphy Type™ Kit (Clondiag, Jena, Germany). Briefly, according to the manufacturer's protocol, two to three inoculation loops of MRSA from blood agar were suspended in 200 µl of a lysis enhancer dissolved in lysis buffer. The suspension was incubated for 30 min at 37ºC and 550 rpm in the thermomixer. The remaining steps were performed using a DNaesy Blood&Tissue Kit (QIAgen, Hilden, Germany) and processed according to the manufacturer's protocol. Briefly, after cell lysis 25 µl proteinase K and 200 µl of buffer AL were added. The solution was shaken and incubated for 30 min at 56ºC and 550 rpm in the thermomixer. 200 µl of absolute ethanol was added and mixed well by pipetting up and down. The solution was transferred into the DNeasy mini spin column placed in a 2 ml collection tube and centrifuged at 8,000 rpm for 1 min. The flow-through and collection tube were discarded and the DNeasy mini spin column was placed into a new 2 ml collection tube. 500 µl of Buffer AW1 was added and centrifuged at 8,000 rpm for 1 min. The flow-through and collection tube were discarded and the DNeasy mini spin column was placed into a new 2 ml collection tube. 500 µl of Buffer AW2 was added and centrifuged at 14,000 rpm for 3 min to dry the DNeasy membrane. The flow-through and collection tube were discarded and the DNeasy mini spin column was placed into a clean 1.5 ml tube. For elution of genomic DNA, 100 µl of Buffer AE was added directly onto the DNeasy membrane and incubated at room temperature for 1 min, and the solution was centrifuged at 8,000 rpm for 1 min. The last step was repeated and subsequently the open tube with 200 µl of genomic DNA solution was placed for 10 min at 70ºC in a thermomixer (remove the ethanol). Finally, the DNA was concentrated by evaporation in a vacuum centrifuge for 1 h to get around 100 µl of genomic DNA solution.

3.2.7.2 DNA labeling and amplification

After preparation of genomic DNA, samples were processed in two further steps, DNA labeling, amplification, hybridization and detection according to the manufacturer's protocol, the Staphy Type™ Kit (Clondiag, Jena, Germany). For DNA labeling and amplification, *S. aureus* DNA was amplified approximately 40-fold and labeled with biotin-dUTP based on the linear PCR protocol. The linear PCR used only one primer which produced single stranded reaction products only. These products limited the degree of

amplification which limited the risk of cross-contamination. For the linear amplification and biotin labeling step, 5 µl of genomic DNA solution was suspended in 5 µl of the Master mix, combining 4.9 µl of labelling buffer B1 and 0.1 µl of DNA polymerase B2. Amplifications were carried out in GeneAmp 2720 Thermal cycles, and the following run condition was used: beginning with pre-heat cover to 105ºC and an initial denaturation step at 96ºC for five minutes follow by 45 cycles of 50ºC for 20 seconds, 72ºC for 30 seconds, 96ºC for 20 seconds, and cool down to 4ºC.

3.2.7.3 DNA hybridization and detection

Labeled, single-stranded DNA was transferred and hybridized to microarrays with 333 probes, including different genetic markers for antimicrobial resistance, production of virulence factors, and information regarding strain assignment. The hybridization and detection step, pre-heated the thermomixer to 55ºC and removed the microarray strip (8 wells) from the bag and placed into the white frame. The microarray strip was washed twice, firstly with ultrapure water by adding 200 µl and mixing carefully with a pipette (4 X up and down) and then removing and discarding the water. In the second washing step, 100 µl of hybridization buffer C1 were added and incubated for 2 min at 55ºC and 550 rpm in the thermomixer. Then the strip was removed and the hybridization buffer C1 discarded. The hybridization mixture was prepared by adding 90 µl of hybridization buffer C1 to each labeled single stranded DNA product (10 µl). Then the hybridization mixture (100 µl) was transferred to a prepared well on the microarray strip. Each well of microarray strip was covered with cap and incubated for 60 min at 55ºC and 550 rpm in the thermomixer.

The microarray strip was removed from the thermomixer, and the thermomixer was set to 30ºC. The cap that covered each well was carefully opened and the hybridization mixture was discarded as complete as possible. After hybridization, each well of the microarray strip was washed twice with washing buffer C2 by adding 200 µl of washing buffer C2 and mixing with a pipette (4 X up and down), and then removing and discarding the washing solution. The addition of Horseradish Peroxidase (HRP)- conjugate step was performed by adding 100 µl of combined reagent C3 (contains Streptavidin-Horseradish Peroxidase, HRP) and Buffer C4 (Reagent C3 : Buffer C4 = 1 : 100) to each well of the microarray strip. It was incubated for 10 min at 30ºC and 550 rpm and the reagent C3 and buffer C4 were removed and discarded. After addition of HRP-conjugate, each well of the microarray strip was washed twice with washing buffer C5 by adding 200 µl of washing

buffer C5 and mixing with a pipette (4 X up and down), and then removing and discarding the washing solution.

Last step was staining of bound HRP-conjugate and reading. To each well 100 µl of reagent D1 (contains a substrate for Horseradish Peroxidase) were added and incubated at room temperature for 5 min. Then it was removed and the reagent D1 discarded as completely as possible and analysed immediately using the ArrayMate Reader (Clondiag, Jena, Germany).

3.2.8 Statistical analysis

The data were analyzed using the SPSS statistic software version 17 (SPSS Inc., Munich, Germany). Descriptive statistic, including frequency, mean, standard deviation, median, minimum, maximum, and Spearman correlation were employed as general analytic procedure to describe the general information on the farms and the occurrence and antimicrobial administered for treatment of selected diseases or health problems in cows and calves. The occurrence of the diseases or health problems in cows and calves were subjective notions of the frequency of the problems (rare = the problems are not happening very often; regular = the problems that frequently happen). The criteria for the usage of antimicrobials in the treatment of diseases or health problems were recorded as never (not at any occasion), sometimes (on some occasions), and frequent (on many occasions).

Chi-square was used to analyze the bivariate association between the ESBLs-producing *E. coli* positive farms and the occurrence of lameness, metritis, dystocia, and surgery in adult cows, the occurrence of respiratory infections, diarrhea, navel ill, and arthritis in calves, and the use of anitimicrobials and 3^{rd} and 4^{th} generation of cephalosporins for treatment of each disease. For multivariate analysis, the variables were tested in a backward stepwise elimination negative binomial regression model, with the ESBLs producing *E. coli* positive finding as dependent variable. A P-value of < 0.05 was considered statistically significant. Due to the limited number of MRSA positive bulk tank milks encountered in the study, no association of these isolates with putative risk factors was analysed.

Chapter 4
Results

In the present study, a questionnaire survey (including questions on farm characteristics, health management in dairy cattle, and treatment strategies emphasizing the usage of antimicrobials) was performed among herd managers of 60 dairy farms located in the regions Brandenburg (n= 18), Lower Saxony and Saxony Anhalt (n= 42) in northern Germany in the period between February and September 2010. The questions were answered by the herd manager, while the form was filled in by the veterinarian during a routine farm visit. Furthermore, bulk tank samples were obtained from the latter farms for bacteriological examination emphasizing MRSA and ESBLs-producing *E. coli*.

4.1 Evaluation of the questionnaire

The dairy herds recruited for the present study had a median size of 218 cows, ranging from 25 to 3000 animals. Table 20 gives an overview over the average herd size, the minimum and maximum herd size, the number of animals in the different lactation groups as well as the number of youngstock on the different farms. For the preceding year the mean culling rate on these farms was 26.6% ranging from 9.3% to 48.7%. Culling rates were positively correlated with herd size. The mean milk yield ranged between 6,190.5 and 10,582.0 kg/year (Table 20). The median bulk milk SCC was 199,000 cells/ml.

Table 20. Descriptive statistics of farm data from 60 dairy farms in northern Germany

	N	Median	Mean	SD	Min	Max
No. of adult cows	60	218	376.6	485.8	25.0	3,000.0
No. of cows in lactation	60	186.0	326.4	416.2	21.0	2,500.0
No. of dry cows	60	30.0	50.2	72.9	2.0	500.0
No. of calves	57	40.0	68.4	91.8	0.0	600.0
No. of heifers	56	85.0	140.5	166.9	0.0	600.0
Culling rate in 2009 (%)	51	25.1	26.6	9.9	9.3	48.7
Milk quota (tons/year)	52	1,800.0	2851.1	4,063.1	60.0	24,000.0
Milk yield /cow /year (kg)	48	8,333.3	8367.1	1,128.0	6,190.5	10,582.0
Bulk tank milk SCC (x1,000 cells per ml)	60	199.0	285.0	460.6	22.0	3,591.0

Table 21 gives an overview over the number of farms, the culling rates, the bulk tank milk SCC, and the average milk yield per cow per year in herds with less than 100 cows (Group 1), herds with 100 to 500 cows (Group 2), and more than 500 cows (Group 3). The culling rates in group 1, group 2, and group 3 were 19.6%, 28.9%, and 30.8%, respectively. The mean annual milk yields in group 1, group 2 and group 3 were 8,133.8 kg, 8,449.3 kg, and 8,429.2 kg, respectively, and the mean bulk milk SCC were 450,680 cells/ml, 213,870 cells/ml, and 250,460 cells/ml, respectively.

Table 21. Descriptive statistics of herd characteristics of 60 dairy farms located in northern Germany obtained by a questionnaire among herd managers. The data are represented for different herd sizes. Group 1: less than 100 cows, Group 2: 100 to 500 cows, Group 3: more than 500 cows

	Herd size		
	Group 1	Group 2	Group 3
Number of farms (%)	16 (26.7)	31 (51.6)	13 (21.7)
Culling rates (%)	19.6	28.9	30.8
Bulk tank milk SCC (cells/ml)	450,680	213,870	250,460
Average milk yield per cow per year (kg)	8,133.8	8,449.3	8,429.2

4.2 Health status with respect to common disorders in dairy cattle

Herd managers from 60 dairies were asked to give an estimation of the occurrence of certain disorders among their cattle by choosing one of the two criteria: rare and regular. The list on the form included disorders occurring in dairy cows and calves that likely require the use of antibacterials. These were lameness, metritis, dystocia, and surgical interventions in cows, and the Bovine Respiratory Disease complex (BRD), diarrhea, diseases of the umbilicus and arthritis in calves. Furthermore, the herd managers were asked to rate the frequency of antibacterial treatments with respect to these disorders on their farms. Results are presented in Tables 22 and 23.

4.2.1 Lameness, dystocia and surgical interventions

On 47.5 % (28 farms) of the dairy farms lameness was rated as a common problem by the herd managers. With respect to cases of dystocia this was true for only 3.6% of the herds

(2 farms). Surgical interventions were performed only incidentally on 89.5% (51 farms) of the farms.

4.2.2 Metritis and mastitis

Metritis was reported to occur only rarely in 72.9 % (43 farms) of the farms. With respect to mastitis the farmers told that on average 2.8% of their cows had to be treated per month; the highest percentage of cows treated per month was 12%. The proportion of cows that had to be treated for mastitis were 5.2%, 4.1%, and 3.5% per month in the herds with less than 100 cows, between 100 and 500 cows, and more than 500 cows, respectively.

Table 22. Herd managers'estimations of the occurrence of lameness, dystocia, metritis and surgical interventions in their herd. Results of a questionnaire among herd managers from 60 dairy farms in northern Germany. Respondents could choose one of the following criteria: rare and regular

Occurrence	Lameness		Metritis		Dystocia		Surgery	
	N	%	N	%	N	%	N	%
rare	31	52.5	43	72.9	53	96.4	51	89.5
regular	28	47.5	16	27.1	2	3.6	6	10.5
No. respondents	59	100.0	59	100.0	55	100.0	57	100.0

Table 23 represents the herd managers' estimations with respect to the usage of antimicrobials in lame cows, cows with metritis, cows with dystocia and in the perioperative management of animals undergoing surgical interventions. The usage of antimicrobials was estimated by herd managers based on the criteria never, sometimes, frequent. Most farmers (67.8%, 40 farms) report frequent administration of antimicrobials in metritis followed by frequent antimicrobial usage in lame cows (50.8%, 30 farms). On 75.9% (41 farms) and 71.2% (37 farms) of farms, respectively, no antimicrobials were administered in case of dystocia or at surgery. On 14.8% of the farms (8 farms), however, antimicrobial drugs were administered to heifers and cows after dystocia.

Table 23. Usage of antimicrobials in the treatment of lameness, metritis, dystocia and in the perioperative management of animals undergoing surgical interventions. Results of a questionnaire among herd managers from 60 dairy farms in northern Germany. Respondents could choose one of the following criteria: never, sometimes, frequent

	Lameness		Metritis		Dystocia		Surgery	
	Number of farms	%	Number of farms	%	Number of farms	%	Number of farms	%
never	18	30.5	8	13.6	41	75.9	37	71.2
sometimes	11	18.6	11	18.6	5	9.3	7	5.8
frequent	30	50.8	40	67.8	8	14.8	12	23.1
respondents	59	100	59	100	54	100	56	100

4.2.3 Occurrence of calf diseases and usage of antimicrobial drugs

Table 24 shows the herd managers' estimations with respect to the occurrence of calf diseases on their farms including the Bovine Respiratory Disease complex (BRD), diarrhea, diseases of the umbilicus and arthritis. BRD and calf diarrhea were the disorders which were reported to occur most frequently on the farms. Diseases of the umbilicus and arthritis were reported to occur only on single occasions.

Table 24. Estimations on the occurrence of calf diseases. Results of a questionnaire among herd managers from 60 dairy farms in northern Germany. Respondents could choose one of the following criteria: rare and regular

Occurrence	BRD		Diarrhea		Diseases of the umbilicus		Arthritis	
	N	%	N	%	N	%	N	%
rare	34	63.0	35	66.0	52	100	52	100
regular	20	37.0	18	34.0	0	0.0	0	0.0
respondents	54	100	53	100	52	100	52	100

The information on antimicrobials administered for treatment of common diseases in calves are shown in Table 25. Herd managers from 83% of the farms (44 farms) answered that antimicrobials were administered to treat calves with BRD, calf diarrhea 61.5% (32

farms), diseases of the umbilicus 42.3% (22 farms), and arthritis 24.5% (13 farms). On nearly two-thirds of the farms (56.6%, 30 farms), antimicrobial agents were most frequently used in the treatment of BRD. 17% of the herd managers (9 farms) reported that antimicrobial agents were never used in the treatment of BRD. With respect to antimicrobial treatment in case of calf diarrhea, 30.8% of the herd managers (16 farms) answered that this was performed frequently while 38.5% (20 farms) reported that on their farms antimicrobial agents were never used in the treatment of calf diarrhea. In addition, 57.7% (30 farms) and 75.5% (40 farms) of the farmers in this study reported that antimicrobial agents were never used for treatment of umbilical disease and arthritis, respectively.

Table 25. Usage of antimicrobials in the treatment of calf diseases. Results of a questionnaire among herd managers from 60 dairy farms in northern Germany. Respondents could choose between one of the following criteria: never, sometimes, frequent

	BRD		Diarrhea		Disease of the umbilicus		Arthritis	
	Number of farms	(%)	Number of farms	(%)	Number of farms	(%)	Number of farms	(%)
never	9	17.0	20	38.5	30	57.7	40	75.5
sometimes	14	26.4	16	30.8	5	9.6	5	9.4
frequent	30	56.6	16	30.8	17	32.7	8	15.1
respondents	53	100	52	100	52	100	53	100

4.3 Udder health

With respect to udder health, herd managers were asked about the decision criteria for posting milk samples to the laboratory requesting a bacteriological examination (Table 26). Herd managers from 70% (42 farms) and 60% (36 farms), respectively, handle clinical disease and abnormal milk as criteria for posting milk samples to the laboratory. On only 13.3% (8 farms) and 20.0% (12 farms) of the farms, respectively, milk samples from cows after parturition and from cows before drying-off were submitted for bacteriological examination on a routine basis. Increased somatic cell counts (SCC) in single cows form a criterion for 45% (27 farms) of the herd managers to send milk samples for bacteriological examination to a laboratory.

Table 26. Herd managers' decision criteria for posting milk samples for bacteriological examination. Results of a questionnaire among herd managers from 60 dairy farms in northern Germany

Criteria for posting milk samples		Number of farms	% of farms
Post calving			
	Yes	8	13.3
	No	52	86.7
	respondents	60	100
High SCC			
	Yes	27	45.0
	No	33	55.0
	respondents	60	100
Before drying-off			
	Yes	12	20.0
	No	48	80.0
	respondents	60	100
Abnormal secreta			
	Yes	36	60.0
	No	24	40.0
	respondents	60	100
Success control following mastitis treatment			
	Yes	6	10.0
	No	54	90.0
	respondents	60	100
Clinical mastitis			
	Yes	42	70.0
	No	18	30.0
	respondents	60	100

On most farms the decision to treat cows affected with mastitis by administration of antimicrobials depends either on the presence of clinical symptoms (58 farms, 100%) in the animal or on the presence of visible alterations of the milk (53 farms, 91.4%) in absence of

other symptoms in the animal. On 16 farms (40%) increased SCC in individual cows gave rise to the use of antibacterials (Table 27).

Table 27. Decision criteria for the use of antibacterials in the treatment of mastitis. Results of a questionnaire among herd managers from 60 dairy farms in northern Germany

Criteria to use antibacterials		Number of farms	% of farms
High SCC in single cows			
	Yes	16	40.0
	No	24	60.0
	respondents	40	100
Visible alterations of the milk only			
	Yes	53	91.4
	No	5	8.6
	respondents	58	100
General condition affected			
	Yes	58	100.0
	No	0	0.0
	respondents	58	100

Table 28 summarizes the management of cows with mastitis as well as dry cow management on the 60 dairy farms. On 30 farms (50%), cows treated for mastitis remain in the group, whereas on 45% (27 farms) such cows were separated from their production group. On three (5%) farms both options are applied. The modalities for treatment of cows affected with mastitis depend on the prescription of the antimicrobial that is used. For this reason the farmers report on different durations in the treatment of mastitis. On nine (16.4%) farms treatment is continued for up to two days. On 38 farms (69.1%) the cows are treated for up to 4 days, and on eight farms (14.5%) treatment is continued for more than four days. On nearly one-third of the farms the duration of treatment depends on the time until visible alterations in the milk have disappeared.

Table 28. Dry cow management and management of cows affected with mastitis. Results of a questionnaire among herd managers from 60 dairy farms in northern Germany

	Number of farms	% of farms
Management of cows treated with antimicrobials		
cows are separated from the group	27	45.0
cows remain in their group	30	50.0
both options are applied	3	5.0
respondents	60	100
Duration of antibacterial treatment in cows with mastitis		
1-2 days	9	16.4
3-4 days	38	69.1
>4 days	8	14.5
respondents	55	100
Treatment continued until milk does not show visible alterations any more		
Yes	19	31.7
No	41	68.3
respondents	60	100
Treatment records for cows with mastitis		
Herd Management Program	15	25.9
Card/Book	34	58.6
Both	9	15.5
respondents	58	100
Dry cow therapy management		
All quarters	51	85.0
Diseased quarters	9	15.0
respondents	60	100

Recordings of mastitis treatments are mandatory in Germany. Farmers are forced to keep the records in a farm recording book. Farmers reported that animal treatments are recorded on cards or in a booklet (34 farms, 58.6%); on 15 farms (25.9%) notes with respect to treatments were documented via the herd management program, and on nine farms

(15.5%) both options were applied. With respect to dry cow therapy, only nine farms (15%) apply a selection of cows that receive a dry cow therapy. On the majority of farms (51 farms, 85%) a routine treatment at drying off is applied, including the intra mammary administration of antimicrobials.

4.4 Correlation analysis

Statistical analysis of correlations between criteria from the questionnaire delivered the following results (Table 29). Positive correlations were found between herd size and culling rates, the occurrence of lameness, the use of antimicrobials in the treatment of lame cows and the use of antimicrobials following dystocia. The occurrence of dystocia was positively correlated with the occurrence of metritis and the use of antimicrobials in the treatment of dystocia.

Table 29. Correlations (Spearmans rho) between herd size, culling rates, occurrence of certain disorders and the usage of antimicrobials. Results of a questionnaire among herd managers from 60 dairy farms in northern Germany

	1	2	3	4	5	6	7	8	9	10
1. Herd size	1									
2.Culling rate	.42**	1								
3. Lameness	.05	.39**	1							
4. Lameness Tx.	.16	.32*	.18	1						
5. Metritis	.27*	.04	.03	.11	1					
6. Metritis Tx.	-.11	.26	.21	.22	.17	1				
7. Dystocia	.25	.16	.01	.05	.35**	.12	1			
8. Dystocia Tx.	.27*	.29*	.19	.23	.18	.32*	.33*	1		
9. Surgery	.09	.25	.15	-.18	.05	-.01	-.06	-.19	1	
10.Perioperative Tx.	.17	.10	-.06	.21	.19	.24	.17	.27	-.01	1

* Correlation is significant at the 0.05 level
** Correlation is significant at the 0.01 level

Table 30 shows the correlations between herd size and the occurrence of calf diseases on one hand and the usage of antimicrobials in calves on the other. Herd size was positively correlated with the use of antimicrobials for treatment of diarrhea and arthritis.

The use of antimicrobials in the treatment of diarrhea was positively correlated with the use of antimicrobials in BRD and the occurrence of diarrhea. In addition, the use of antimicrobials for treatment of arthritis was positively correlated with the use of antimicrobials for treatment of umbilical diseases.

Table 30. Correlations (Spearmans rho) between herd size and the occurrence of calf diseases and the usage of antimicrobials in the treatment of these diseases. Results of a questionnaire among herd managers from 60 dairy farms in northern Germany

	1	2	3	4	5	6	7	8	9
1. Herd size	1								
2. BRD	.31*	1							
3. BRD Tx.	.01	.26	1						
4. Diarrhea	.27*	.05	.06	1					
5. Diarrhea Tx.	.34*	.16	.28*	.35*	1				
6. Umbilical diseases	[a]	[a]	[a]	[a]	[a]	[a]			
7. Tx of umbilical diseases	.28*	.10	.29*	.17	.08	[a]	1		
8. Arthritis	[a]	[a]	[a]	[a]	[a]	[a]	[a]	[a]	
9. Tx of Arthritis	.53**	.14	.14	-.01	.11	[a]	.44**	[a]	1

* Correlation is significant at the 0.05 level
** Correlation is significant at the 0.01 level
[a] Cannot be computed because at least one of the variables is constant.

Table 31 shows the correlations between herd size, culling rates, treated cows/month, management of cows treated for mastitis or due to high SCC, and decision criteria for sending milk samples for bacteriological examination and for treatment of mastitis. The routine to separate cows following treatment with antibacterials from the rest of the group was positively correlated with the herd size. The same was true for the routine to post milk samples from post calving cows for bacterial culture. Posting milk samples from freshly calved cows for bacteriological examination as well as the control of treatment success following the treatment of cows with mastitis were positively correlated to the culling rates. A positive correlation was found between the routine to post milk samples from cows that reveal abnormal secreta and the administration of antimicrobials in the treatment of mastitis.

Table 31. Correlations (Spearmans rho) between herd size, culling rates, % treated cows with mastitis/month, management of cows treated with antibacterials, and decision criteria for posting milk samples for bacteriological examination and for treatment of mastitis. Results of a questionnaire among herd managers from 60 dairy farms in northern Germany

	1	2	3	4	5	6	7	8	9	10	11	12
1. Herd size	1											
2. % culling cow/year	.42**	1										
3. % Tx. Mastitic cow/month	-.13	.10	1									
4. Culture post calving	.36**	.46**	.17	1								
5. Culture HiSCC	.06	.03	-.18	-.26*	1							
6. Culture before dry-off	-.22	-.12	-.001	.17	-.20	1						
7. Culture abnormal milk	-.07	.15	.30*	.12	-.15	-.19	1					
8. Culture after mastitis Tx.	.25	.48**	.18	.20	.15	-.17	.27*	1				
9. Culture clinical mastitis	-.18	.10	.17	-.06	-.21	-.04	.43**	.10	1			
10. Tx. HiSCC	-.07	.24	.14	.16	.04	.16	-.12	-.04	.16	1		
11. Tx. Abnormal milk	.07	.13	.20	-.06	.16	-.17	.27*	.10	.06	.15	1	
12. Treated cow management	.66**	.24	.07	.39**	.03	-.08	.00	.22	-.07	.12	.07	1

* Correlation is significant at the 0.05 level
** Correlation is significant at the 0.01 level

4.5 Usage of antimicrobial drug classes

Table 32 shows the usage of different antimicrobial drug classes in the treatment of different disorders in cows and calves on 60 dairy farms in northern Germany. The antimicrobial drug classes administered to cows included beta-lactams, tetracyclines, macrolides, sulfonamides, and fluoroquinolones. Amoxicillin+clavulanic acid was the only fixed drug combination that made part of antibacterial treatments in adult cows.

Table 32. List of antimicrobial drug usage for treatment of different disorders in cows and calves on 60 dairy farms in northern Germany

Antimicrobial drug class	Antimicrobials used in cows	Antimicrobials used in calves
Beta-Lactams	Penicillin	Penicillin
	Amoxicillin	Cloxacillin
	Cephapirin (1st generation)	Amoxicillin
	Ceftiofur (3rd generation)	Ceftiofur (3rd generation)
	Cefquinome (4th generation)	Cefquinome (4th generation)
Tetracycline	Tetracycline	Tetracycline
	Oxytetracycline	Oxytetracycline
	Chlortetracycline	
Macrolide	Erythromycin	Tylosin
		Tilmicosin
		Tulathromycin
Sulfonamide	Sulfamethoxazole	Sulfamethoxazole
Fluoroquinolone	Danofloxacin	Danofloxacin
		Enrofloxacin
		Marbofloxacin
Aminoglycoside		Neomycin
		Streptomycin
		Gentamicin
Amphenicol		Florfenicol
Polypeptide		Colistin
Drug combinations	Amoxicillin+Clavulanic acid	Amoxicillin+Clavulanic acid
		Ampicillin+Cloxacillin
		Penicillin+Streptomycin

Beta-lactams, tetracyclines, macrolides, sulfonamides, fluoroquinolones, aminoglycocides, derivatives of chloramphenicol and polypeptides were the antimicrobial drug classes applied in the treatment of common calf diseases (Table 25). Amoxicillin+clavulanic acid, amoxicillin+cloxacillin, and penicillin+streptomycin were the fixed drug combinations that were administered to calves.

4.6 Antimicrobial agents administered to adult dairy cows

Ceftiofur was administered on 55.9% of the farms (33 farms) to lame cows. Whenever herd managers reported that antimicrobials were used on a routine basis in the treatment of lame cows (80.5% of the farms), ceftiofur was most frequently administered. Metritis was treated with either amoxicillin (27.1% of all farms, 31.4% of farms applying antibacterial agents), ceftiofur (18.6% of all farms, 21.6% of farms applying antimicrobial agents), and/or tetracyclines (27.1% of all farms, 31.4% of farms applying antimicrobial agents). The herd managers of two farms reported the use of solely non-steroidal antiinflammatory drugs (NSAID) in the treatment of metritis (Table 33).

Table 33. Usage of antimicrobials in the treatment of lameness and metritis. Results of a questionnaire among herd managers of 60 dairy farms in northern Germany

Antimicrobial	Lameness			Metritis		
	N	% of all farms	% of farms applying antimicrobials	N	% of all farms	% of farms applying antimicrobials
Penicillin	6	10.2	14.6	6	10.2	11.8
Amoxicillin	5	8.5	12.2	16	27.1	31.4
Amoxicillin+Clavulanic acid	0	0.0	0.0	2	3.4	3.9
Ampicillin+Cloxacillin	0	0.0	0.0	1	1.7	1.9
Cephapirin	0	0.0	0.0	3	5.1	5.9
Ceftiofur	33	55.9	80.5	11	18.6	21.6
Cefquinome	2	3.4	4.9	5	8.5	9.8
Fluoroquinolone	1	1.7	2.4	2	3.4	3.9
Tetracycline	2	3.4	4.9	16	27.1	31.4
Sulfonamide/ Trimethoprim+Sulfonamide	0	0.0	0.0	1	1.7	1.9
Other	0	0.0	0.0	2	3.4	3.9

Penicillin was administered on 9.1% of all farms (38.5% of farms applying antimicrobials) to cows subsequently to dystocia. Cephapirin was used in such cows on 3.6 % of all farms, which makes 15.4% of the farms applying antimicrobials to cows after dystocia. The antimicrobial agents preferentially used in the perioperative treatments were

penicillin (10.5 % of all farms, 31.6% of farms applying antimicrobial agents) and ceftiofur (8.8 % of all farms, 26.3% of farms applying antimicrobial agents). On one farm exclusively NSAID's were administered to cows after dystocia or following surgery (Table 34).

Table 34. Antimicrobials administered to cows following dystocia and in the regimen of the perioperative management. Results of a questionnaire among herd managers of 60 dairy farms in northern Germany

	Dystocia			Surgery		
	N	% of all farms	% of farms applying antimicrobials	N	% of all farms	% of farms applying antimicrobials
Penicillin	5	9.1	38.5	6	10.5	31.6
Amoxicillin	1	1.8	7.7	1	1.8	5.3
Cephapirin	2	3.6	15.4	0	0.0	0.0
Ceftiofur	1	1.8	7.7	5	8.8	26.3
Cefquinome	1	1.8	7.7	2	3.5	10.5
Fluoroquinolone	1	1.8	7.7	0	0.0	0.0
Tetracycline	1	1.8	7.7	0	0.0	0.0
Macrolide	1	1.8	7.7	0	0.0	0.0
Other	1	1.8	7.7	1	1.8	5.3

4.7 Antimicrobial agents administered to calves

Table 35 shows antimicrobial usage in calves in the treatment of BRD and calf diarrhea on 60 dairy farms in northern Germany. Antimicrobial agents preferentially used to treat calves suffering from BRD were florfenicol (35.2% of all farms, 43.2% of farms applying antimicrobials in BRD) and macrolides (tilmicosin and tulathromycin) (25.9% of all farms, 31.2% of farms applying antimicrobials in BRD). Herd managers from six farms (11.1% of all farms, 13.6% of farms applying antimicrobials in BRD) reported that they used solely NSAID's to treat BRD. Quinolones (danofloxacin, enrofloxacin, and marbofloxacin) (28.3% of all farms, 46.9% of farms applying antimicrobials in calves with diarrhea) were the most frequently used antimicrobial agents to treat calf diarrhea. Ten farmers (18.9% of all farms, 31.2% of farms applying antimicrobial agents in calves with diarrhea) reported that on their farms exclusively NSAID are used in the treatment of calf diarrhea.

Table 35. Antimicrobials used in the treatment of BRD and calf diarrhea. Results of a questionnaire among herd managers of 60 dairy farms in northern Germany

	BRD			Calf Diarrhea		
	N	% of all farms	% of farms applying antimicrobials	N	% of all farms	% of farms applying antimicrobials
Penicillin	3	5.6	6.8	2	3.8	6.3
Penicillin+Streptomycin	1	1.8	2.3	0	0.0	0.0
Amoxicillin	1	1.8	2.3	1	1.9	3.1
Amoxicillin+Clavulanic acid	1	1.8	2.3	1	1.9	3.1
Cloxacillin	1	1.8	2.3	0	0.0	0.0
Ampicillin+Cloxacillin	1	1.8	2.3	0	0.0	0.0
Cefquinome	1	1.8	2.3	4	7.5	12.5
Florfenicol	19	35.2	43.2.	1	1.9	3.1
Fluoroquinolone	4	7.2	9.1	15	28.3	46.9
Tetracyclines	1	1.8	2.3	0	0.0	0.0
Macrolides	14	25.9	31.2	2	3.8	6.3
Aminoglycosides	1	1.8	2.3	1	1.9	3.1
Sulfonamides	0	0.0	0.0	1	1.9	3.1
Other	6	11.1	13.6	10	18.9	31.2

Umbilical infections were most frequently treated with penicillin (25.0% of all farms, 59.1% of farms applying antimicrobial agents in calves with umbilical diseases). Penicillin (11.5% of all farms, 46.2% of farms applying antimicrobial agents in calves with arthritis), amoxicillin (3.8% of all farms, 15.4% of farms applying antimicrobial agents in calves with arthritis), ceftiofur (3.8% of all farms, 15.4% of farms applying antimicrobial agents in calves with arthritis), and fluoroquinolones (3.8% of all farms, 15.4% of farms applying antimicrobial agents in calves with arthritis) are the commonly used antimicrobial agents in the treatment of arthritis. With respect to umbilical diseases, one farm reported the exclusive administration of NSAID whereas two farmers reported that solely NSAID were used in the treatment of arthritis (Table 36).

Table 36. Antimicrobials used in the treatment of umbilical diseases and arthritis. Results of a questionnaire among herd managers of 60 dairy farms in northern Germany

	Umbilical diseases			Arthritis		
	N	% of all farms	% of farms applying antimicrobials	N	% of all farms	% of farms applying antimicrobials
Penicillin	13	25.0	59.1	6	11.5	46.2
Penicillin+Streptomycin	3	5.8	13.6	0	0.0	0.0
Amoxicillin	1	1.9	4.5	2	3.8	15.4
Amoxicillin+Clavulanic acid	1	1.9	4.5	0	0.0	0.0
Ceftiofur	1	1.9	4.5	2	3.8	15.4
Fluoroquinolone	1	1.9	4.5	2	3.8	15.4
Tetracycline	0	0.0	0.0	1	1.9	7.7
Other	1	1.9	4.5	2	3.8	15.4

4.8 Antimicrobial agents administered to cows with mastitis and management at drying-off

Table 37 shows the use of different drug classes in the treatment of mastitis and in dry cow management on the 60 dairy farms in northern Germany. Beta-lactam antibiotics (penicillin, ampicillin, amoxicillin, cloxacillin, oxacillin, cefuroxime, cefoperazone, and cefquinome), tetracyclines, macrolides (erythromycin and tylosin), lincosamide, and fluoroquinolones (danofloxacin, enrofloxacin, and marbofloxacin) were applied on the latter farms. Combinations of antimicrobial agents applied in the treatment of mastitis or in dry cow therapy included cefalexin+kanamycin, ampicillin+cloxacillin, lincomycin+neomycin, amoxicillin with clavulanic acid, lincomycin+amoxicillin, penicillin+fluoroquinolone, and penicillin+neomycin. The latter combinations are fixed drug combinations in products registered for treatment of mastitis. Beta-lactams (cloxacillin, oxacillin, cephapirin, and cefquinome) and fixed drug combinations (penethamate hydriodide + benethamine penicillin + framycetinsulfate) were the antimicrobial drugs used at drying-off.

Table 37. List of antimicrobials used in the treatment of mastitis and in dry cow treatment. Results of a questionnaire among herd managers on 60 dairy farms in northern Germany

Antimicrobial drug class	Antimicrobial drugs for treatment of mastitis	Antimicrobial drugs for dry cow therapy
Beta-Lactams	Penicillin	Cloxacillin
	Ampicillin	Oxacillin
	Amoxicillin	Cephapirin (1st generation)
	Cloxacillin	Cefquinome (4th generation)
	Oxacillin	
	Cefuroxime (2nd generation)	
	Cefoperazone (3rd generation)	
	Cefquinome (4th generation)	
Tetracyclines	Tetracycline	
Macrolides	Erythromycin	
	Tylosin	
Lincosamide	Lincomycin	
Fluoroquinolone	Danofloxacin	
	Enrofloxacin	
	Marbofloxacin	
Drugs combination	Amoxicillin+Clavulanic acid	Penethamate
	Cefalexin(1st generation)	hydriodide+benethamine
	+Kanamycin	penicillin+framycetinsulfate
	Ampicillin+Cloxacillin	Cloxacillin+Teat sealant
	Lincomycin+Neomycin	Cefquinome (4th generation)
	Lincomycin+Amoxicillin	+Teat sealant
	Penicillin+Fluoroquinolone	
	Penicillin+Neomycin	
Non antimicrobial	Homeopathic	Internal Teat Sealant

4.9 Usage of antimicrobial agents in the treatment of mastitis and dry cow therapy on the different farms

Table 38 shows antimicrobial agent usage in the treatment of mastitis and in dry cow therapy. Cefquinome (4th generation cephalosporin) was the most frequently used antimicrobial agent (81.7% of the farms) administered intramammarily in case of mastitis. Penicillin (40.0%), lincosamide (23.3%), and fluoroquinolones (20.0%) were antimicrobial agents that were also used to treat mastitis. Frequently used fixed drug combinations were

Table 38. Antimicrobial usage in the treatment of mastitis. Results of a questionnaire among herd managers of 60 farms in northern Germany

Therapeutics		Treatment of mastitis	
		Number of farms	%
Penicillin		24	40.0
Ampicillin		1	1.7
Amoxicillin		2	3.3
Cloxacillin		1	1.7
Oxacillin		7	11.7
Cefuroxime (2nd Generation)		1	1.7
Cefperazone (3rd Generation)		6	10.0
Cefquinome (4th Generation)		49	81.7
Macrolide		5	8.3
Lincosamide		14	23.3
Fluoroquinolone		12	20.0
Tetracycline		1	1.7
Drug combinations	Cefalexin+Kanamycin	8	13.3
	Ampicillin+Cloxacillin	6	10.0
	Amoxicillin+Clavulanic acid	5	8.3
	Lincomycin+Neomycin	1	1.7
	Lincomycin+Amoxicillin	1	1.7
	Penicillin+Fluoroquinolone	2	3.3
	Penicillin+Neomycin	1	1.7
Homeopathic		4	6.7
Other		7	11.7

cefalexin+kanamycin (13.3%), ampicillin+cloxacillin (10.0%), and amoxicillin+clavulanic acid (8.3%). Four herd managers reported the use of homeopathic products and on seven farms solely NSAID's were used in the treatment of mastitis.

The most common antimicrobials administered intramammarily at drying-off included penethamate hydriodide+benethamine penicillin+framycetinsulfate (29 farms, 48.3%), cloxacillin (23 farms, 38.3%) and cefquinome (9 farms, 15.0%). On 17 dairy farms (28.3%) solely internal teat sealants were used at drying-off (Table 39).

Table 39. Usage of antimicrobial agents and teat sealants at drying off. Results of a questionnaire among herd managers of 60 dairy farms in northern Germany

	Dry cow therapy	
	Number of farms	%
Penethamate hydriodide+benethamine penicillin+framycetinsulfate	29	48.3
Cloxacillin	23	38.3
Oxacillin	2	3.3
Cephapirin (1st Generation)	1	1.7
Cefquinome (4th Generation)	9	15.0
Cefquinome+Internal Teat Sealant	1	1.7
Cloxacillin+Internal Teat Sealant	2	3.3
Internal Teat Sealant	17	28.3

4.10 Detection of Methicillin-resistant *Staphylococcus aureus* (MRSA)

Bacterial culture and efforts to isolate MRSA from bulk tank milk derived from 60 dairy farms delivered positive results in bulk tank milk samples obtained from four (6.7%) farms. As mentioned in the Materials and Methods section, besides the samples obtained from the 60 farms, isolates from milk samples stored at the BFR were also characterized by a molecular biological approach. A detailed characterization of the 36 MRSA strains isolated from milk in this study is shown in Table 40.

Table 40. Characterization data for 36 MRSA strains derived from milk samples -among these four bulk tank samples from the dairy farms included in the study

Number	Sample ID	Multiplex PCR			SCC*mec* type	*spa* type	Strain
		16s	*nuc* gene	*mec*A gene			
1	09S-160	+	+	+	*mec* V	t011	ST398
2	09S-161	+	+	+	*mec* V	t011	ST398
3	09S-162	+	+	+	*mec* V	t011	ST398
4	09S-196	+	+	+	*mec* V	t011	ST398
5	09S-531	+	+	+	*mec* V	t011	ST398
6	09S-534	+	+	+	*mec* V	t034	ST398
7	09S-535	+	+	+	*mec* V	t034	ST398
8	09S-627	+	+	+	*mec* V	t034	ST398
9	09S-629	+	+	+	*mec* V	t034	ST398
10	09S-1065	+	+	+	*mec* V	t011	ST398
11	09S-1066	+	+	+	*mec* V	t011	ST398
12	09S-1076	+	+	+	*mec* V	t034	ST398
13	09S-1088	+	+	+	*mec* V	t011	ST398
14	09S-1380	+	+	+	*mec* III	t034	ST398
15	09S-1386	+	+	+	*mec* III	t034	ST398
16	09S-1391	+	+	+	*mec* V	t011	ST398
17	09S-1659	+	+	+	*mec* V	t011	ST398
18	09S-1770	+	+	+	*mec* V	t034	ST398
19	09S-1772	+	+	+	*mec* V	t011	ST398
20	09S-1774	+	+	+	*mec* V	t011	ST398
21	09S-1776	+	+	+	*mec* V	t011	ST398
22	09S-2339	+	+	+	*mec* V	t011	ST398
23	09S-2376	+	+	+	*mec* V	t011	ST398
24	10S-423	+	+	+	*mec* V	t034	ST398
25	10S-428	+	+	+	*mec* V	t034	ST398
26	10S-500	+	+	+	*mec* V	t011	ST398
27	10S-969	+	+	+	*mec* V	t011	ST398
28	10S-1200	+	+	+	*mec* V	t011	ST398
29	10S-1237	+	+	+	*mec* V	t011	ST398
30	10S-1253	+	+	+	*mec* IVa	t011	ST398
31	10S-1399	+	+	+	*mec* V	t011	ST398
32	H07-1	+	+	+	*mec* V	t034	ST398
33	H07-2	+	+	+	*mec* V	t034	ST398
34	H18	+	+	+	*mec* V	t011	ST398
35	H34	+	+	+	*mec* V	t034	ST398
36	H40	+	+	+	*mec* V	t034	ST398

In total, 36 MRSA isolates from bovine milk were tested in the present study. Five isolates from the MRSA positive strains (number 32 to 36) originated from the sampling on the dairy farms included in the present study and 31 isolates (number 1 to 31) originated from the Bundesinstitut für Risikobewertung (BfR or Federal Institute for Risk Assessment) and included 13 isolates from a national monitoring project on bulk tank milk (ZoMo 2009). 18 further bulk tank milk isolates were submitted to the National Reference Laboratory (NRL) in 2009 and 2010 in the framework of other projects.

All 36 isolates were proven to be MRSA by multiplex PCR and DNA micro array analysis which were shown positive for the 16S rDNA specific for *Staphylococcus* species, the *S. aureus*- specific region of the thermonuclease gene (*nuc*), and the resistance gene *mec*A. Two different *spa* types were identified, including t011 and t034, and the dominant type was t011 (n = 22). For SCC*mec* typing, 33 strains had SCC*mec* type V, 2 strains had SCC*mec* type III, and only one strain had SCC*mec* type IVa. From DNA micro array analysis, all 36 MRSA strains from bovine milk in this study were ST398.

4.11 Antimicrobial resistance of 36 MRSA strains

All 36 MRSA strains isolated from bovine milk were tested against 13 antimicrobial agents by the broth micro dilution method. Table 41 shows the results of antimicrobial susceptibility testing. Resistance to oxacillin (OXA) and tetracycline (TET) was demonstrated for all strains isolated from milk. Furthermore, the majority of strains revealed a resistance to clindamycin (CLI), erythromycin (ERY), kanamycin (KAN), and quinupristin/dalfopristin (QUI/DAL). This was true for 21 (58.3%), 19 (52.8%), 10 (27.8%), and 13 (36.1%) of the isolates, respectively. Resistance to ciprofloxacin (CIP) was found in three isolates (8.3%). The MIC_{90} of gentamicin, quinupristin/dalfopristin (QUI/DAL), ciprofloxacin, and sulfamethoxazole+trimethoprim were 64 µg/ml, 4 µg/ml, 1 µg/ml, and 19+1 µg/ml, respectively. In the present study, only one strain was resistant to chloramphenicol. The MIC_{90} value was at 16µg/ml. The MIC_{90} for clindamycin, erythromycin, kanamycin, oxacillin and tetracycline cannot be reported as they were above the tested range of concentrations. All 36 MRSA strains isolated from bovine milk in this study were susceptible to mupirocin (MUP), vancomycin (VAN), and linezolid (LZD). The MIC_{90} for mupirocin, vancomycin, and linezolid were 1 µg/ml, 2 µg/ml, and 4 µg/ml, respectively.

Table 41. Minimum inhibitory concentrations (MIC) of 13 different antimicrobial agents versus 36 strains of MRSA isolated from milk

Antimicrobial agent	MIC (µg/ml)											Resistant[a]	
	0.25	0.5	1	2	4	8	16	32	64	128	256	No.	%
CHL				-	-	12	**23**	-	1	-	-	1	2.8
CIP		28	**5**	2	-	-	1	-	-			3	8.3
CLI	15	3	-	-	-	-	4	3	11			21	58.3
ERY	3	12	2	-	-	-	-	19				19	52.8
KAN						26	4	1	-	1	4	10	27.8
MUP			**36**	-	-	-	-					0	0
OXA+2%NaCl			-	-	2	8	26					36	100
QUI/DAL		14	9	6	**4**	2	1					13	36.1
TET			-	-	-	-	-	-	7	29		36	100
VAN				**36**	-	-	-	-				0	0
LZD			2	12	**22**	-	-					0	0
GEN		22	7	1	-	1	1	-	**4**			6	16.7
SXT[b]	21	10	**3**	2	-	-	-					5	13.9

CHL – Chloramphenicol, CIP – Ciprofloxacin, CLI – Clidamycin, ERY – Erythromycin, KAN – Kanamycin, MUP – Mupirocin, OXA – Oxacillin, QUI/DAL – Quinupristin/Dalfopristin, TET – Tetracycline, VAN – Vancomycin, LZD – Linezolid, GEN – Gentamicin, SXT – Sulfamethoxazole/Trimethoprim

The MIC values that inhibit half of the isolates (MIC_{50}) are underlined.

The MIC values that inhibit 90% of the isolates (MIC_{90}) are shown in bold.

[a] Isolates were classified as resistant after EUCAST epidemiological cut-off value (black vertical lines) for MRSA and/or *S. aureus* valid at the time of submission.

[b] The MIC values of sulfamethoxazole/trimethoprim (19:1) are given as trimethoprim MIC values.

Dilution ranges tested are marked in white. Values above the range indicate MIC values > the highest concentration in the range. Values at the lowest concentration tested indicate MIC value ≤ the lowest concentration in the range.

Table 42 shows the antimicrobial resistance patterns of the 36 MRSA strains derived from milk samples. In total, 16 resistance patterns were detected. The most common resistance pattern was TET-OXA alone which was found in 10 strains (27.8%). Multidrug resistance (MDR) to a range from three to seven antimicrobial agents was found in 26 (72.2%) strains, and the most predominant multidrug resistance pattern was TET-ERY-CLI-OXA-QUI/DAL (25.0%). Quinupristin/Dalfopristin (QUI/DAL) resistance occurred in 13 isolates (36.1%) and was associated with resistance to four or more antimicrobial agents. Resistance to trimethoprim/sulfamethoxazole (SXT) was observed in five strains (13.9%), and only one strain was resistant to chloramphenicol.

Table 42. Detection rates of antimicrobial resistance phenotypes in 36 MRSA strains derived from milk samples

Resistance phenotype	Numbers	%
TET OXA	10	27.8
TET ERY CLI OXA QUI/DAL	9	25.0
KAN TET OXA	2	5.6
TET ERY CLI OXA	2	5.6
GEN KAN TET CLI OXA	2	5.6
GEN KAN TET OXA SXT	1	2.8
TET CLI OXA	1	2.8
TET OXA SXT	1	2.8
TET CIP ERY OXA	1	2.8
KAN TET ERY CLI OXA	1	2.8
KAN TET ERY CLI OXA QUI/DAL	1	2.8
TET ERY CLI OXA QUI/DAL SXT	1	2.8
TET CIP ERY CLI OXA QUI/DAL CHL	1	2.8
GEN KAN TET ERY CLI OXA QUI/DAL	1	2.8
GEN KAN TET ERY CLI OXA SXT	1	2.8
GEN KAN TET CIP ERY CLI OXA SXT	1	2.8
Total	36	100

CHL – Chloramphenicol, CIP – Ciprofloxacin, CLI – Clidamycin,
ERY – Erythromycin, GEN – Gentamicin, KAN – Kanamycin,
OXA – Oxacillin, QUI/DAL – Quinupristin/Dalfopristin,
TET – Tetracycline, SXT – Trimethoprim/Sulfamethoxazole

4.12 DNA Microarray analysis

4.12.1 Genes encoding for antimicrobial resistance

Genes encoding antimicrobial resistance of 36 MRSA strains isolated from bovine milk were analysed by DNA microarray analysis (Table 43). All MRSA strains in this study carried more than one antimicrobial resistance gene. Eighteen different antimicrobial resistance gene patterns were identified. One MRSA isolate carried four of the investigated antimicrobial resistance genes (2.8%), three carried six genes (8.3%), 16 carried seven genes (44.4%), 12 carried eight genes (33.3%), three carried nine genes (8.3%), and one isolate carried ten antimicrobial resistance genes (2.8%). The most common genotypic

resistance patterns were *mec*A-*blaZ*-*bla*I-*bla*R-*tet*M-*tet*Efflux-*tet*K 8 strains (22.2%), *mec*A-*blaZ*-*bla*I-*bla*R-*tet*M-*tet*Efflux-*erm*A 5 strains (13.9%), *mec*A-*blaZ*-*bla*I-*bla*R-*tet*M-*tet*Efflux 3 strains (8.3%), and *mec*A-*blaZ*-*bla*I-*bla*R-*tet*M-*tet*Efflux-*tet*K-*erm*A 3 strains (8.3%).

Table 43. Antimicrobial resistance genes demonstrated in 36 MRSA strains derived from bovine milk samples

Genes	No. of positive isolates	%
*mec*A, *tet*M, *tet*Efflux, *tet*K	1	2.8
*mec*A, *blaZ*, *bla*I, *bla*R, *tet*M, *tet*Efflux	3	8.3
*mec*A, *blaZ*, *bla*I, *bla*R, *tet*M, *tet*Efflux, *erm*A	5	13.9
*mec*A, *blaZ*, *bla*I, *bla*R, *tet*M, *tet*Efflux, *erm*C	1	2.8
*mec*A, *blaZ*, *bla*I, *bla*R, *tet*M, *tet*Efflux, *aac*A-*aph*D	2	5.6
*mec*A, *blaZ*, *bla*I, *bla*R, *tet*M, *tet*Efflux, *erm*A, *aad*D	1	2.8
*mec*A, *blaZ*, *bla*I, *bla*R, *tet*M, *tet*Efflux, *erm*A, *erm*C	1	2.8
*mec*A, *blaZ*, *bla*I, *bla*R, *tet*M, *tet*Efflux, *erm*B, *aad*D	2	5.6
*mec*A, *blaZ*, *bla*I, *bla*R, *tet*M, *tet*Efflux, *erm*C, *aad*D, *fex*A	1	2.8
*mec*A, *blaZ*, *bla*I, *bla*R, *tet*M, *tet*Efflux, *vga*A, *aac*A-*aph*D	1	2.8
*mec*A, *blaZ*, *bla*I, *bla*R, *tet*M, *tet*Efflux, *tet*K	8	22.2
*mec*A, *blaZ*, *bla*I, *bla*R, *tet*M, *tet*Efflux, *tet*K, *erm*A	3	8.3
*mec*A, *blaZ*, *bla*I, *bla*R, *tet*M, *tet*Efflux, *tet*K, *erm*C	2	5.6
*mec*A, *blaZ*, *bla*I, *bla*R, *tet*M, *tet*Efflux, *tet*K, *vga*A	1	2.8
*mec*A, *blaZ*, *bla*I, *bla*R, *tet*M, *tet*Efflux, *tet*K, *erm*A, *erm*B	1	2.8
*mec*A, *blaZ*, *bla*I, *bla*R, *tet*M, *tet*Efflux, *tet*K, *erm*C, *vga*A	1	2.8
*mec*A, *blaZ*, *bla*I, *bla*R, *tet*M, *tet*Efflux, *tet*K, *vga*A, *aac*A-*aph*D	1	2.8
*mec*A, *blaZ*, *bla*I, *bla*R, *tet*M, *tet*Efflux, *tet*K, *erm*C, *vga*A, *aac*A-*aph*D	1	2.8
Total	36	100

All 36 MRSA strains isolated from bovine milk carried *mec*A, *tet*M, and *tet*Efflux antimicrobial resistance genes. Antimicrobial resistance genes *blaZ*, *bla*I, *bla*R, *tet*K, and *erm*A were detected in high frequency in 97.2, 97.2, 97.2, 52.8, and 30.6 % of isolates, respectively. Fewer MRSA strains carried *erm*B (8.3%), *erm*C (19.4%), *vga*A (13.9%), *aac*A-*aph*D (13.9%), and *aad*D (11.1%). Only one isolate (2.8%) of MRSA from milk

sample in this study carried the *fex*A antimicrobial resistance gene (Table 44). All 36 MRSA strains tested negative for *van*A, *van*B, *van*Z (vancomycin), *msr*A, *mef*A, *mpb*BM (macrolides), *lin*A, *cfr* (lincosamides), *vat*A, *vat*B, *vga*, *vgb* (streptogramin), *aph*A (aminoglycoside), *sat* (streptothricin), *dfr*A (trimethoprim), *far* (fusidic acid), *mup*R (mupirocin), and *cat* (chloramphenicol) (Appendix C).

Table 44. Percentage of genes encoding antimicrobial resistance of 36 MRSA strains derived from milk as demonstrated by DNA microarray analysis

Resistance gene	N	%	Explanation
*mec*A	36	100.0	Methicillin, Oxacillin and all Beta-lactams, defining MRSA
*bla*Z	35	97.2	Beta-Lactamase
*bla*I	35	97.2	Beta-Lactamase repressor (regulatory protein)
*bla*R	35	97.2	Beta-Lactamase regulatory protein
*erm*A	11	30.6	Macrolide, Lincosamide, Streptogramin
*erm*B	3	8.3	Macrolide, Lincosamide, Streptogramin
*erm*C	7	19.4	Macrolide, Lincosamide, Streptogramin
*vga*A	5	13.9	Streptogramin
*aac*A-*aph*D	5	13.9	Aminoglycoside (Gentamycin, Tobramycin)
*aad*D	4	11.1	Aminoglycoside (Tobramycin, Neomycin)
*tet*K	19	52.8	Tetracycline
*tet*M	36	100	Tetracycline
*tet*Efflux	36	100	Tetracycline Efflux Protein (Putative transport protein)
*fex*A	1	2.8	Chloramphenicol

Table 45 shows the information on antimicrobial resistance in pheno- and genotypes of the 36 MRSA strains isolated from bovine milk. In most cases, the results of antimicrobial resistance genes obtained from DNA microarray analysis were in accordance with the findings of phenotypic susceptibility testing. All 36 MRSA ST398 strains harbored genes encoding resistance to methicillin, oxacillin, and all beta-lactams (*mec*A), and 35 strains (97.2%) harbored *bla*I, *bla*Z, *bla*R (beta-lactamase). All strains that carried genes encoding resistance to tetracycline (*tet*M and *tet*Efflux) also revealed phenotypic resistance to tetracycline. For 13 MRSA strains that showed phenotypic resistance to quinupristin/dalfopristin, eight strains harbored *erm*A, one strain harbored *erm*C or *erm*A and *erm*B or *erm*A and *erm*C, and two strains harbored *erm*C and *vga*A. Additionally, one

strain that showed resistance to chloramphenicol in the phenotype also carried the gene encoding resistance to chloramphenicol (*fex*A).

Table 45. Resistance genes and gene expression in 36 MRSA strains derived from samples of bovine milk

ID	Resistance Pattern	Resistance gene
09S-160	KAN TET OXA	*mec*A *bla*Z *bla*I *bla*R *tet*M *tet*Efflux *tet*K
09S-161	TET OXA	*mec*A *bla*Z *bla*I *bla*R *tet*M *tet*Efflux *tet*K *vga*A *aac*A-*aph*D
09S-162	GEN KAN TET CLI OXA	*mec*A *bla*Z *bla*I *bla*R *tet*M *tet*Efflux *tet*K
09S-196	KAN TET ERY CLI OXA	*mec*A *bla*Z *bla*I *bla*R *tet*M *tet*Efflux *tet*K *erm*C
09S-531	GEN KAN TET CLI OXA	*mec*A *bla*Z *bla*I *bla*R *tet*M *tet*Efflux *vga*A *aac*A-*aph*D
09S-534	TET ERY CLI OXA QUI/DAL	*mec*A *bla*Z *bla*I *bla*R *tet*M *tet*Efflux *tet*K *erm*A
09S-535	TET ERY CLI OXA QUI/DAL	*mec*A *bla*Z *bla*I *bla*R *tet*M *tet*Efflux *erm*A
09S-627	TET ERY CLI OXA QUI/DAL	*mec*A *bla*Z *bla*I *bla*R *tet*M *tet*Efflux *erm*A
09S-629	TET OXA	*mec*A *bla*Z *bla*I *bla*R *tet*M *tet*Efflux
09S-1065	KAN TET OXA	*mec*A *bla*Z *bla*I *bla*R *tet*M *tet*Efflux *aac*A-*aph*D
09S-1066	TET OXA	*mec*A *bla*Z *bla*I *bla*R *tet*M *tet*Efflux
09S-1076	TET CIP ERY OXA	*mec*A *bla*Z *bla*I *bla*R *tet*M *tet*Efflux *erm*A
09S-1088	TET OXA	*mec*A *bla*Z *bla*I *bla*R *tet*M *tet*Efflux *tet*K
09S-1380	KAN TET ERY CLI OXA QUI/DAL	*mec*A *bla*Z *bla*I *bla*R *tet*M *tet*Efflux *erm*A *aad*D
09S-1386	TET ERY CLI OXA QUI/DAL	*mec*A *bla*Z *bla*I *bla*R *tet*M *tet*Efflux *erm*A *erm*C
09S-1391	TET OXA	*mec*A *tet*M *tet*Efflux *tet*K
09S-1659	TET OXA	*mec*A *bla*Z *bla*I *bla*R *tet*M *tet*Efflux *tet*K
09S-1770	TET ERY CLI OXA QUI/DAL	*mec*A *bla*Z *bla*I *bla*R *tet*M *tet*Efflux *erm*A
09S-1772	TET OXA	*mec*A *bla*Z *bla*I *bla*R *tet*M *tet*Efflux *tet*K
09S-1774	TET ERY CLI OXA	*mec*A *bla*Z *bla*I *bla*R *tet*M *tet*Efflux *erm*C
09S-1776	TET OXA	*mec*A *bla*Z *bla*I *bla*R *tet*M *tet*Efflux
09S-2339	TET ERY CLI OXA	*mec*A *bla*Z *bla*I *bla*R *tet*M *tet*Efflux *tet*K *erm*C
09S-2376	TET ERY CLI OXA QUI/DAL	*mec*A *bla*Z *bla*I *bla*R *tet*M *tet*Efflux *tet*K *erm*C *vga*A
10S-423	TET ERY CLI OXA QUI/DAL	*mec*A *bla*Z *bla*I *bla*R *tet*M *tet*Efflux *tet*K *erm*A
10S-428	TET ERY CLI OXA QUI/DAL	*mec*A *bla*Z *bla*I *bla*R *tet*M *tet*Efflux *tet*K *erm*A
10S-500	GEN KAN TET CIP ERY CLI OXA SXT	*mec*A *bla*Z *bla*I *bla*R *tet*M *tet*Efflux *erm*B *aad*D
10S-969	GEN KAN TET ERY CLI OXA SXT	*mec*A *bla*Z *bla*I *bla*R *tet*M *tet*Efflux *erm*B *aad*D
10S-1200	TET OXA	*mec*A *bla*Z *bla*I *bla*R *tet*M *tet*Efflux *tet*K
10S-1237	TET CLI OXA	*mec*A *bla*Z *bla*I *bla*R *tet*M *tet*Efflux *tet*K *vga*A
10S-1253	GEN KAN TET OXA SXT	*mec*A *bla*Z *bla*I *bla*R *tet*M *tet*Efflux *aac*A-*aph*D
10S-1399	GEN KAN TET ERY CLI OXA QUI/DAL	*mec*A *bla*Z *bla*I *bla*R *tet*M *tet*Efflux *tet*K *erm*C *vga*A *aac*A-*aph*D
H07-1	TET OXA SXT	*mec*A *bla*Z *bla*I *bla*R *tet*M *tet*Efflux *tet*K
H07-2	TET ERY CLI OXA QUI/DAL SXT	*mec*A *bla*Z *bla*I *bla*R *tet*M *tet*Efflux *tet*K *erm*A *erm*B
H18	TET OXA	*mec*A *bla*Z *bla*I *bla*R *tet*M *tet*Efflux *tet*K
H34	TET CIP ERY CLI OXA QUI/DAL CHL	*mec*A *bla*Z *bla*I *bla*R *tet*M *tet*Efflux *erm*C *aad*D *fex*A
H40	TET ERY CLI OXA QUI/DAL	*mec*A *bla*Z *bla*I *bla*R *tet*M *tet*Efflux *erm*A

CHL – Chloramphenicol, CIP – Ciprofloxacin, CLI – Clidamycin, ERY – Erythromycin, GEN – Gentamicin, KAN – Kanamycin, OXA – Oxacillin, QUI/DAL – Quinupristin/Dalfopristin, TET – Tetracycline, SXT – Trimethoprim/Sulfamethoxazole

Few inconsistencies were observed with respect to the detection of genes encoding for antimicrobial resistance. None of the strains in this study carried the gene encoding resistance to trimethoprim (*dfr*A) while for five strains phenotypic resistance to trimethoprim/sulfamethoxazole was demonstrated. In contrast ten strains revealed phenotypic resistance to kanamycin while the genes for aminoglycoside resistance (*aac*A-*aph*D, *aad*D, *aph*A) were detected only in seven strains. Furthermore, from 21 strains that showed combined phenotypic resistance to erythromycin and clindamycin, in 18 strains the genes for marcolides and lincosemide resistance (*erm*A, *erm*B, *erm*C) were detected but none of the strains harbored *lin*A and *cfr* (Lincosamide).

4.12.2 Genes encoding for enterotoxins, toxic shock syndrome toxins, leukocidins, and hemolysins

Table 46 shows information on genes encoding enterotoxins, toxic shock syndrome toxins, leukocidins, and hemolysins in the 36 MRSA strains isolated from bovine milk by DNA microarray analysis. In the present study, all strains tested negative for all of genes encoding enterotoxins (Appendix C). In addition, genes encoding toxic shock syndrome toxins (*tst*1, *tst*-RF122), Pantone-Valentine Leukocidin (PVL), leukocidins (*luk*M/*luk*F-P83, *luk*D, *luk*E, *luk*Y-var2), and hemolysin Beta (hlb), were found in none of all strains isolated from the bovine milk in the present study. For the genes encoding leukocidins/haemolysin toxin family protein, the DNA microarray analysis showed *luk*X and *luk*Y-var1 to be present in all strains in this study. In addition, all 36 MRSA ST398 strains harbored *hla* (haemolysin alpha), un-truncated *hlb* (haemolysin beta), *hld* (haemolysin delta), *hl* (hypothetical protein similar to haemolysin), *hl*III, *hl*_III_other than RF122 (putative haemolysin III), and *luk*F, *luk*S, *hlg*A (haemolysin gamma).

Table 46. Percentage of genes encoding enterotoxin, toxic shock syndrome toxins, leukocidins, and hemolysins of 36 MRSA strains from milk by DNA microarray analysis

Gene	N	%	Explanation
tst-1	0	0	Toxic shock syndrome toxin
tst-RF122	0	0	Toxic shock syndrome toxin, allele from bovine strains
PVL	0	0	Pantone-Valentine Leukocidin
*luk*M/lukF-P83	0	0	Bovine Leukocidin
*luk*F	36	100	Haemolysin Gamma, Component B
*luk*S	36	100	Haemolysin Gamma, Component C
*luk*S-ST22+ST45	2	5.6	Haemolysin Gamma, Component C, allele from ST22 and ST45
*hlg*A	36	100	Haemolysin Gamma, Component A
*luk*D	0	0	Leukocidin D Component
*luk*E	0	0	Leukocidin E Component
*luk*X	36	100	Leukocidin/Haemolysin Toxin Family Protein
*luk*Y-var1	36	100	Leukocidin/Haemolysin Toxin Family Protein
*luk*Y-var2	0	0	Leukocidin/Haemolysin Toxin Family Protein, allele from MRSA252
hl	36	100	Hypothetical Protein similar to Haemolysin
hla	36	100	Haemolysin Alpha (Alpha Toxin)
hld	36	100	Haemolysin Delta (Amphiphylic Membrane Toxin)
*hl*III	36	100	Putative Haemolysin III
*hl*_III_Other than RF122	36	100	Putative Haemolysin III (Other than RF122)
hlb	0	0	Haemolysin Beta (Phospholipase C)
Un-truncated *hlb*	36	100	Haemolysin Beta (Phospholipase C/ un-truncated)

4.13 Extended-Spectrum Beta-Lactamases producing *E. coli* (ESBLs-producing *E. coli*)

In 18 (30%) bulk tank samples that originated from the 60 dairy farms participating in the present study, ESBLs-producing *E. coli* were detected (Table 47). With respect to the number of animals on the farms the distribution of samples being demonstrated positive for ESBLs-producing *E. coli* was as follows: 12.5% (2 farms) of farms with less than 100 cows, 32.3% (10 farms) of herds with 100 to 500 cows, and 46.2% (6 farms) of herds with more than 500 cows. The difference was not significant.

Table 47. Isolation of ESBLs-producing coliforms from bulk tank milk samples originating from 60 dairy farms in northern Germany

	Number of farms	%
ESBLs Coliform bacteria	32	53.3
- *E. coli*	18	30.0
- *Hafnia alvei*	6	10.0
- *Enterobacter cloacae*	4	6.7
- *Klebsiella pneumonia*	2	3.3
- *Citrobacter braakii/freundii*	2	3.3

4.13.1 Herd health and detection of ESBLs-producing *E. coli* in bulk tank samples

Table 48 gives an overview over the results of the questionnaire emphasizing the relation between the presence of ESBLs-producing *E. coli* and the herd managers' estimations of the occurrence of common diseases in their dairy herds. On 18 farms with bulk tank samples being tested positive for ESBLs- producing *E. coli*, lameness was reported as the most frequent disorder observed in dairy cows (55.5 % of farms). Approximately 11% (2 farms) of farms with positive test results reported that dystocia is a major problem in their herd. In the latter herds surgical interventions are performed on a regular basis. 50% (9 farms) of farms with positive bulk tank samples reported BRD as a major problem in calves, whereas none of the latter reported the occurrence of umbilical disease and arthritis as a major problem.

Table 48. Herd managers' estimations of the occurrence of distinct diseases and disorders on their farms and the detection of ESBLs-producing *E. coli* in bulk tank samples obtained at one occasion

	ESBLs *E. coli* positive			ESBLs *E. coli* negative			Chi-square	P value
	N	Missing data	% regular occurrence	N	Missing data	% regular occurrence		
Disorders in cows								
Lameness	18	0	55.5	41	1	43.9	0.681	0.409
Metritis	18	0	44.4	41	1	19.5	3.934	0.047
Dystocia	17	1	11.8	38	4	0	4.639	0.031
Surgery	18	0	11.1	39	3	10.3	0.010	0.922
Disorders in calves								
BRD	18	0	50.0	36	6	30.6	1.946	0.163
Diarrhea	18	0	44.4	35	7	28.6	1.335	0.248
Umbilical Disease	18	0	0	34	8	0	[a]	[a]
Arthritis	18	0	0	34	8	0	[a]	[a]

[a] No statistics were computed

In ESBLs-producing *E. coli* negative farms (farms with a negative test result), lameness and metritis often occurred in adult cows in 43.9% and 19.5% of farms, respectively. Approximately one-third of the farms found that BRD and diarrhea occurred regularly in their calves. In this study, there were no statistically significant differences between farms with positive and negative test results with respect to the occurrence of most of the diseases and disorders included in the questionnaire. However, the detection of ESBLs-producing *E. coli* in bulk tank milk was associated with the occurrence of metritis and dystocia in adult cows ($p < 0.05$).

4.13.2 Usage of antimicrobials on farms with bulk tank milk samples positive for ESBL-producing *E. coli*

Table 49 shows the association of ESBLs-producing *E. coli* tested positive farms and the use of antimicrobials in adult cows and in calves. 82.4% (14 farms) and 88.9% (16 farms) of farms with a positive test results used antimicrobials for treatment of lameness and metritis in cows, respectively. In contrast, only 5.4% of farms (2 farms) testing negative for ESBLs-

producing *E. coli* at sampling on a single occasion used antimicrobials for the treatment of cows following dystocia.

Table 49. Detection of ESBLs-producing *E. coli* in bulk tank milk at sampling on a single occasion related to the herd managers' information on the use of antimicrobials on 60 dairy farms included in the study

	ESBLs positive			ESBLs negative			Chi-square	P value
	N	Missing data	% of farms applying antimicrobials	N	Missing data	% of farms applying antimicrobials		
Disorders in cows								
Lameness	17	1	82.4	42	0	64.3	1.863	0.172
Metritis	18	0	88.9	40	2	87.5	0.023	0.881
Dystocia	17	1	64.7	37	5	5.4	22.410	0.000
Surgery	15	3	40.0	37	5	24.3	1.278	0.258
Disorders in calves								
BRD	17	1	82.4	36	6	83.3	0.008	0.929
Diarrhea	17	1	64.7	35	7	60.0	0.107	0.744
Umbilical Diseases	17	1	64.7	35	7	31.4	5.191	0.023
Arthritis	17	1	47.1	36	6	13.9	6.863	0.009

Farms with positive and negative test results usually used antimicrobials for treatment of BRD in calves, but only 31.4% (11 farms) and 13.9 % (5 farms) of negative test result farms used antimicrobials for treatment of umbilical diseases and arthritis as compared to 64.7% (11 farms) and 47.1 % (8 farms) in positive test result farms, respectively. The use of antimicrobials for treatment of dystocia in adult cows and navel ill and arthritis in calves was associated with the detection of ESBLs-producing *E. coli* in bulk tank milk ($p < 0.05$). In the multivariate analysis (backward stepwise elimination negative binomial regression model), we found that the use of antimicrobials in the treatment of cows after dystocia was associated with the detection of ESBLs-producing *E. coli* in bulk tank milk of the farms ($p < 0.05$).

4.13.3 Detection of ESBLs-producing *E. coli* in bulk tank milk at sampling on a single occasion in relation to herd managers' information on the use of 3rd and 4th generation of cephalosporins

Table 50 shows the association of ESBLs-producing *E. coli* positive bulk tank milk samples and the use of 3rd and 4th generation of cephalosporins on the 60 farms included in the study.

Table 50. Association of ESBLs-producing *E. coli* in bulk tank milk obtained at sampling on a single occasion and the use of 3rd and 4th generation cephalosporins on the 60 dairy farms included in this study

	ESBLs positive			ESBLs negative			Chi-square	P value
	N	Missing data	% treatment with 3rd & 4th*	N	Missing data	% treatment with 3rd & 4th*		
Disorders in cows								
Lameness	17	1	64.7	42	0	57.1	0.287	0.592
Metritis	18	0	27.8	40	2	25.0	0.050	0.823
Dystocia	17	1	5.9	37	5	0	2.218	0.136
Surgery	15	3	13.3	37	5	13.5	0.000	0.986
Disorders in calves								
BRD	17	1	0	36	6	2.8	0.481	0.488
Diarrhea	17	1	11.8	35	7	5.7	0.590	0.442
Umbilical Diseases	17	1	0	35	7	2.9	0.495	0.482
Arthritis	17	1	5.9	36	6	2.8	0.307	0.580

* Use of 3rd and 4th generation of cepharosporins for treatment of diseases or health problems

In this study, 72.2% (13 farms) of test positive farms and 69.0% (29 farms) of test negative farms used 3rd and 4th generation cephalosporins for treatment of diseases or health problems in adult cows and in calves. The use of 3rd and 4th generation cephalosporins for treatment of diseases or health problems in adult cows and in calves was not associated with the detection of ESBLs-producing *E. coli* in bulk tank milk sampled at a single occasion ($p = 0.806$). In 64.7% (11 farms) and 27.8% (5 farms) of the farms with positive test results, 3rd and 4th generation cephalosporins were used to treat lameness and metritis in adult cows, respectively. Only 5.9% (1 farm) of positive test result farms used 3rd and 4th generation cephalosporins to treat cows after dystocia. None of the test negative farms treated dystocia

with 3rd and 4th generation cephalosporins, and 57.1% (24 farms) and 25 % (10 farms) of the farms used 3rd and 4th generation cephalosporins for the treatment of lameness and metritis, respectively. None of the positive farms used 3rd and 4th generation cephalosporins for treatment of respiratory infections and navel ill in calves, and positive and negative farms rarely used 3rd and 4th generation cephalosporins for treatment of diseases or health problems in their calves.

4.14 Antimicrobial resistance of ESBLs-producing *E. coli*

In the present study, all 18 ESBLs-producing *E. coli* strains from bulk tank milk were tested against 15 standard antimicrobial agents for enterobacteria and against 15 beta-lactam antimicrobial agents. Table 51 shows the results of antimicrobial susceptibility testing against 15 standard antimicrobial agents for enterobacteria of all the 18 ESBLs-producing *E. coli* strains in this study.

Table 51. Susceptibility of 18 ESBLs-producing *E. coli* strains isolated from bulk tank milk in the 60 dairy farms to 15 standard antimicrobial agents. Figures in brackets indicate inhibition zone diameters in intermediate isolates

No.	Sample ID	Standard antimicrobial agents for enterobacteria														
		AMP 10	CHL 30	FLO 30	GEN 10	KAN 30	CIP 5	NAL 30	AMC 30	TET 30	S 10	SPE 100	SU 300	TMP 5	SXT 25	EFT 30
1	01	R	-	-	-	-	-	-	-	-	-	-	-	-	-	R
2	02	R	-	-	-	-	-	R	-	R	-	-	-	R	R	R
3	03	R	-	-	-	-	-	-	-	-	-	-	-	-	-	R
4	05	R	R	-	-	R	-	-	-	R	R	-	R	-	-	R
5	14	R	-	-	-	-	-	-	-	-	-	-	-	-	-	R
6	15	R	-	-	-	-	-	-	-	-	R	-	R	-	-	R
7	H07	R	-	-	R	R	R	R	R	R	R	-	R	R	R	R
8	H17	R	-	-	-	-	-	-	-	-	-	-	-	-	-	R
9	H19	R	-	-	R	R	R	R	I(15)	I(14)	-	-	R	R	R	R
10	H20	R	-	-	-	-	-	-	-	-	-	-	-	-	-	R
11	H21	R	-	-	R	-	R	R	-	R	R	-	R	-	-	R
12	H24	R	R	-	-	-	-	-	-	R	I(13)	-	R	-	-	R
13	H25	R	-	-	-	R	R	R	I(15)	R	-	-	-	-	-	R
14	H29	R	-	-	-	-	-	-	-	-	-	-	-	-	-	R
15	H30	R	-	-	-	-	-	-	-	-	-	-	-	-	-	R
16	H32	R	-	-	-	-	-	-	-	-	-	-	-	-	-	R
17	H34	R	-	-	-	-	-	-	-	-	-	-	-	-	-	R
18	H40	R	-	-	-	-	R	R	-	R	R	I(13)	R	R	R	R

AMP – Ampicillin, CHL – Chloramphenicol, FLO – Florfenicol, GEN – Gentamicin, KAN – Kanamycin, CIP – Ciprofloxacin, NAL – Nalidixic acid, AMC – Amoxicillin+Clavulanic acid, TET – Tetracycline, S – Streptomycin, SPE – Spectinomycin, SU – Sulfonamide, TMP – Trimethoprim, SXT - Trimethoprim/Sulfamethoxazole, EFT – Ceftiofur
R – resistant, I – intermediate, - susceptible

All strains isolated from bulk tank milk obtained at sampling on a single occasion in this study were found resistant to ampicillin (AMP) and ceftiofur (EFT). Resistance to ciprofloxacin (CIP), nalidixic acid (NAL), tetracycline (TET), streptomycin (S), and sulfonamide (SU) was detected in 27.8%, 33.3%, 38.9%, 27.8%, and 38.9% of isolates, respectively. Resistance to chloramphenicol (CHL) was found in 2 isolates (11.1%) and only one strain was resistant to amoxicillin+clavulanic acid (AMC). All ESBLs-producing *E. coli* strains isolated from bulk tank milk in this study were susceptible to florfenicol (FLO) and spectinomycin (SPE) (Table 52).

Table 52. Susceptibility of 18 ESBLs-producing *E. coli* strains to 15 standard antimicrobial agents. The strains were isolated from bulk tank milk obtained at sampling on a single occasion from 60 dairy farms

Antimicrobial agent	Number (%) of resistant strains (n = 18)
Ampicillin	18 (100)
Chloramphenicol	2 (11.1)
Florfenicol	0 (0)
Gentamicin	3 (16.7)
Kanamycin	4 (22.2)
Ciprofloxacin	5 (27.8)
Nalidixic acid	6 (33.3)
Amoxicillin + Clavulanic acid	1 (5.6)
Tetracycline	7 (38.9)
Streptomycin	5 (27.8)
Spectinomycin	0 (0)
Sulfonamide	7 (38.9)
Trimethoprim	4 (22.2)
Trimethoprim/Sulfamethoxazole	4 (22.2)
Ceftiofur	18 (100)

Table 53 shows the results of antimicrobial susceptibility testing against 15 beta-lactam antimicrobial agents of ESBLs-producing *E. coli* strains.

Table 53. Susceptibility to 15 beta-lactam antimicrobial agents as determined by disk diffusion of 18 ESBLs-producing *E. coli* strains isolated from bulk tank milk obtained at sampling on a single occasion from 60 dairy farms in northern Germany. Figures in brackets indicate inhibition zone diameters in intermediate isolates

No.	Sample ID	Beta-lactam antimicrobial agents														
		AMP 10	CEF 30	CXM 30	TIC 75	PIP 100	EFT 30	CRO 30	IMP 10	CTX 30	AMC 30	CAZ 30	AZM 30	CPD 10	FOX 30	FEP 30
1	01	R	R	R	R	R	R	R	R	R	-	-	I(19)	R	-	R
2	02	R	R	R	R	R	R	R	-	R	-	-	I(19)	R	-	R
3	03	R	R	R	R	R	R	R	-	R	-	-	I(20)	R	-	R
4	05	R	R	I(15)	R	R	I(20)	R	-	R	-	-	-	R	-	-
5	14	R	R	R	R	R	R	R	-	R	-	-	I(18)	R	-	R
6	15	R	R	R	R	R	R	R	-	R	-	-	-	R	-	R
7	H07	R	R	R	R	R	R	R	-	I(15)	I(16)	-	I(18)	R	-	R
8	H17	R	R	R	R	R	R	R	-	R	-	-	-	R	-	I(16)
9	H19	R	R	R	R	R	R	R	-	R	I(15)	I(17)	R	R	-	R
10	H20	R	R	R	R	R	R	R	-	I(16)	-	-	-	R	-	I(17)
11	H21	R	R	R	R	R	R	R	-	I(15)	-	-	I(18)	R	-	I(17)
12	H24	R	R	R	R	R	R	R	-	I(16)	-	-	I(20)	R	-	R
13	H25	R	R	R	R	R	R	R	-	R	-	-	I(18)	R	-	I(17)
14	H29	R	R	R	R	R	R	I(15)	-	I(21)	-	-	-	R	-	-
15	H30	R	R	R	R	R	R	R	-	R	-	-	I(20)	R	-	I(15)
16	H32	R	R	R	R	R	R	R	-	I(16)	-	-	-	R	-	-
17	H34	R	R	R	R	R	R	R	-	I(18)	-	-	-	R	-	-
18	H40	R	R	R	R	R	R	R	-	I(16)	-	-	-	R	-	-

AMP – Ampicillin, CEF – Cephalothin, CXM – Cefuroxime,TIC – Ticarcillin, PIP – Piperacillin,
EFT – Ceftiofur, CRO – Ceftriaxone, IMP – Imipenem, CTX – Cefotaxime,
AMC - Amoxicillin+Clavulanic acid, CAZ – Ceftazidime, AZM – Aztreonam , CPD – Cefpodoxime ,
FOX – Cefoxitin , FEP – Cefepime
R – resistant, I – intermediate, - susceptible

All strains isolated from bulk tank milk in this study were resistant to ampicillin, cephalothin, ticarcillin, piperacillin, ceftiofur, and cefpodoxime. None of the ESBLs-producing *E. coli* strains was resistant to amoxicillin + clavulanic acid, ceftazidime and cefoxitin, and 94.4% of the strains were resistant to cefuroxime, ceftiofur, and ceftriaxone. Resistance to imipenem and aztreonam was detected in only 5.6% of the strains (Table 54).

Table 54. Susceptibility of 18 ESBLs-producing *E. coli* strains to 15 beta-lactam antimicrobial agents. The strains were isolated from bulk tank milk samples obtained at sampling on a single occasion from 60 dairy farms in northern Germany

Antimicrobial agent	Number (%) of resistant strains (n = 18)
Ampicillin	18 (100)
Cephalothin	18 (100)
Cefuroxime	17 (94.4)
Ticarcillin	18 (100)
Piperacillin	18 (100)
Ceftiofur	17 (94.4)
Ceftriaxone	17 (94.4)
Imipenem	1 (5.6)
Cefotaxime	10 (55.6)
Amoxicillin + Clavulanic acid	0 (0)
Ceftazidime	0 (0)
Aztreonam	1 (5.6)
Cefpodoxime	18 (100)
Cefoxitin	0 (0)
Cefepime	8 (44.4)

Chapter 5
Discussion

The occurrence of severe nosocomial infections in hospitalized patients caused by multiresistant bacteria such as MRSA and ESBLs-producing coliforms has drawn the attention of the public to potential sources of the latter bacteria. Besides the practice of prescribing antibacterials in man without too much reflection on the possible consequences, veterinary medicine came into the focus of the public as antibacterials are widely used in the treatment of various disorders affecting small animals, horses, ruminants, pigs, and poultry. On one hand potential risks arise from the close contact between man and companion animals; on the other hand products derived from food producing animals could harbor multirestistant bacteria and form a potential hazard for the consumer, especially juvenile, elderly or immune suppressed individuals. MRSA have been isolated from patients (horses, small animals) and staff members of animal hospitals as well as from staff members and animals from farms where pigs and veal calves are kept (Khanna et al., 2008; Walther et al., 2008; Graveland et al., 2010; van Duijkeren et al., 2010).

The present study should deliver insights into the usage of antibacterials on dairy farms in Germany and focus on the presence of MRSA and ESBLs-producing *E. coli* in bulk tank milk samples. To this end a questionnaire was performed among herd managers of a convenience sample of 60 dairy farms located in northern Germany who were willing to participate in the study. On a single occasion bulk tank milk samples were obtained for bacteriological examination including further characterization of MRSA and ESBLs-producing *E. coli*. These samples were obtained at the same day when the questionnaire was performed.

5.1 Herd size, milk yields and culling rates

The farms included in the present study were located in three regions of northern Germany (Brandenburg, Saxony-Anhalt, Lower Saxony). The typical farm located in Lower Saxony - in contrast to the other two provinces - is a family-run dairy with about 60 to 200 dairy cows, whereas the farms located in the other two provinces are mostly large dairy operations employing a variable number of workers for the care of the animals. Due to the different types and intensities of farming the results of the questionnaire demonstrate huge differences with respect to average milk yields per year, ranging from 6,190 to 10,582 l/y.

These differences can be explained by the different conditions under which milk is produced on the various farms. Major effects on the yearly milk yield are related to genetics, production management and housing conditions. Holstein Friesian cattle - in comparison to cattle of other breeds - have a higher potential to produce large amounts of milk (White et al., 2002; Prendiville et al., 2010). In addition, the production system has a substantial effect on milk yield. As a rule, organic dairy farms - due to restrictions associated with this type of farming - have lower milk yields than conventional dairy farms (Pieper, 2011). Cattle kept in confined housing systems all through the year tend to produce greater amounts of milk than cattle that are pastured during the summer (White et al., 2002).

The culling rates were higher on large dairy operations compared to smaller, mainly family-run dairy farms. Animal observation has been shown to be deficient in larger herds compared to family-run farms. As a consequence of the decreasing milk price the number of workers was reduced on the large dairy operations in the recent years. The remaining staff members have less time for animal observation and care on those farms. In addition, as a consequence of the low wages that are paid in the agricultural sector nowadays well-trained personnel is scarce. For this reason animal observation and care is limited, which contributes to higher culling rates on larger dairy operations compared to smaller family-run farms. The assumption, however, that high milk yields cause an increase in the culling rates (Wangler, 2010) cannot be verified for farms included in this study. Many animals leave the dairy at an age of two to three years. These losses have been reported to originate from diseases that occur as a consequence of maladaptation in heifers in their first lactation (Wangler, 2010). In all age groups, most cows leave the farm within the first month following calving (Wangler, 2010). This finding indicates the crucial role of housing conditions and management of the cow around calving.

5.2 Occurrence of common diseases and antimicrobial usage in cows

In the perception of herd managers taking part in the present questionnaire mastitis, lameness and metritis are the predominant disorders observed on their farms. These findings are in accordance with data reported from dairy farms in northern Germany (Wangler, 2010) as well as from USA (Sawant et al., 2005). A study in 113 dairy herds in Pennsylvania (USA) demonstrated that foot rot and metritis were the most commonly observed disease conditions in lactating cows. A study on economic losses caused by common diseases on 90 dairy farms in England estimated the cost of ill health in a 100 cows herd at about £6300 per

year (Kossaibati and Esslemont, 1997). Main losses were caused by mastitis and lameness. Foot rot has been cited as a frequent health problem by dairy producers surveyed in Canada (Spicer et al., 1994). Lewis (1997) indicated that cows with dystocia and retained placenta are more likely to develop metritis. Likewise, in this study the occurrence of dystocia was associated with the occurrence of metritis.

In Germany, antimicrobials may be used in animals only when prescribed by a veterinarian. The German Veterinary Chamber has issued a guidline in order to enhance the prudent usage of antibacterials in food producing animals (Bundestierärztekammer, 2010). Thus the questionnaire reflects the routine use of antibiotics as prescribed by the veterinarian on the farms. The present study has demonstrated that the administration of antimicrobial drugs is a routine treatment of lame cows and cows affected with metritis on most dairy farms as well. The use of antibacterials in lame cows only makes sense when the animals suffer from distinct infectious claw diseases e.g. interdigital phlegmona or digital dermatitis (Cook and Cutler, 1995). Far more success can be booked with respect to lameness by improving the environmental conditions - mainly the flooring and the cubicles - and by introducing functional claw trimming on a farm (Toussaint Raven, 1993; Cook et al., 2004). Following dystocia or in perioperative management, in contrast, no routine antibacterial treatment was applied on the majority of farms. On large dairy operations the farmers perform most of the obstetrical interventions on their own without calling the veterinarian. However, dystocia has been shown to be associated with post partum disorders (metritis) whenever the vaginal tract is contaminated during assisted calving (Potter et al., 2010).

The treatment of left abomasal displacement - a common disorder in dairy cows - using the roll and toggle procedure (Grymer and Sterner, 1982) does not per se require antibacterial treatment. For this reason herd mangers report that no antibacterials are used at surgery on their farms. Perioperative use of antibacterials at abdominal surgery has been issue of controversial discussions among cattle practitioners. Administration of antibacterials via the intramuscular route or topical application of such drugs to exposed surfaces and body cavities make only sense if adequate concentrations of the drug are guaranteed, which is unlikely in most cases (Brumbauch, 1990). Although there are only scarce reports on the antibacterial management of surgery in cattle (Klein and Firth, 1988), there is persuading evidence - mainly derived from studies in humans and in other animal species - that the administration of antibiotics should take place two to one hour preceding

surgery (Brumbauch, 1990). Based on the findings of the clinical examination and at surgery the veterinarian, however, has to decide, whether antibacterial treatment should be continued in the period following surgery or not.

Considering the different classes of antibacterial drugs, the herd managers report that beta-lactam antibiotics, tetracyclines, macrolides, sulfonamides, and fluoroquinolones are predominantly administered to sick cows on their farms. These findings are in accordance with reports from USA (Brumbauch, 1990). In a study on 90 conventional farms located in Michigan, Minnesota, New York, and Wisconsin the most commonly used antimicrobial agents for treatment of foot rot were ceftiofur (58.6%), penicillin (42.2%), and tetracyclines (24.2%), and for treatment of retained placenta or metritis, penicillin (43.4%) and ceftiofur (41.4%) (Zwald et al., 2004). A study in dairy herds in Pennsylvania reports that sulfadimethoxine (27.3%) and ceftiofur (15%) are the most common antimicrobial agents used in the treatment of foot rot and metritis, respectively (Sawant et al., 2005).

In general, antimicrobials may only be used for the treatement of diseases when licensed for the condition in the respective animal species. Penicillin, ceftiofur, and cefquinome are approved for use in the treatment of interdigital phlegmona and/or digital dermatitis in cattle in Germany. Although penicillin and tetracyclines have been shown to be effective in the treatment of most infectious diseases of the digit, ceftiofur is preferentially administered to lame cows for economic reasons, because ceftiofur does not require a withdrawal period for milk. The same is true for the use of ceftiofur in the treatment of metritis. In addition, the high concentrations in the uterine lumen following parenteral administration of ceftiofur have been shown to deliver more favorable results than local treatments (Drillich et al., 2006). Amoxicillin and tetracyclines that were also mentioned as common therapeutics in the treatment of metritis are preferentially administered locally in the first days after calving. The usage of these antibacterials, although a withdrawal period for milk is demanded, will not cause an economical disadvantage as milk from the first five days after parturition may not be marketed anyway. Penicillin and cephapirin were the commonly used antimicrobial agents to treat cows following dystocia, and penicillin and ceftiofur were frequently used for treatment of cows at surgery. As penicillin is efficient in the treatment of anaerobic agents and ceftiofur also controls infections caused by gram-negative bacteria, these antibacterials are judged suitable for use at surgery (Brumbauch, 1990).

5.3 Occurrence of common diseases and antimicrobial usage in calves

Respiratory diseases and calf diarrhea were the disorders which were most frequently observed in young stock on the farms taking part in the present study. Umbilical diseases and arthritis were reported to occur only sporadically. In accordance with reports from the US (Sawant et al., 2005) and Europe (Ortman and Svensson, 2004), pneumonia and enteritis are the predominant disease conditions that occur in dairy and beef calves. In Pennsylvania, 28 (88%) and 33 farms (100%) reported clinical cases of pneumonia and enteritis, respectively (Sawant et al., 2005). In heifer calves from 112 Swedish dairy herds, respiratory disease and diarrhea were the most common disease conditions occurring in the period from birth up to an age of 210 days (Ortman and Svensson, 2004).

Herd managers report that primarily BRD (83% of the farms) was treated with antimicrobial agents, whereas the treatment protocol for calf diarrhea included an antibacterial treatment only on 61.5% of the farms. For umbilical disease this was true for 42.3% of the farms and for arthritis for 24.5%. BRD and neonatal calf diarrhea are multifactorial diseases. These diseases occur when various factors including infectious agents, environmental conditions and the condition of the animal act together.

Calf diarrhea is a common problem on dairy farms and associated with a wide range of causative agents. The latter include rotaviruses, coronaviruses, Bovine Virus Diarrhea Virus, cryptosporidia, enterotoxicogenic *E. coli*, salmonella and coccidia (Hunt, 1985). The need for antibacterial treatment in the therapy of calf diarrhea is discussed controversially (Constable, 2004). The administration of rehydration solutions in order to compensate for losses of water, electrolytes, glucose and bicarbonate, however, is considered the most important treatment in diarrheic calves. About 30% of diarrheic calves are septicemic and thus need antibacterial treatment (Constable, 2004). The latter author concludes in his review article that antibacterials do not necessarily need to be included in standard protocols for treatment of calf diarrhea. The decision, however, to administer antibacterials should be made on basis of the clinical findings in the diseased animals.

BRD is a condition occurring in calves on dairy and beef cattle farms. Stress, insufficient ventilation, infectious agents, the crowding phenomenon and the higher susceptibility of the bovine respiratory tract predispose calves for diseases of the respiratory tract. In the initial stage of BRD various agents (viruses, bacteria including mycoplasma) - some of which are also normal inhabitants of the upper respiratory tract - colonize the lower respiratory tract and elicit an inflammatory response (Panciera, 2010). After a certain stage

has been passed, BRD is self-supporting. Due to an insufficient first line defense of the upper respiratory tract, dust particles loaded with bacteria reach the deeper airways. The bacteria may cause severe tissue damage and lead to the formation of abscesses (Chirino-Trejo and Prescott, 1983).

On the farms included in the study the veterinarians administer various antibacterials. The herd managers report on the usage of beta-lactam antibiotics, tetracyclines, macrolides, sulfonamides, fluoquinolones, aminoglycosides, florfenicol, and polypeptides. Fluoroquinolones (danofloxacin, enrofloxacin, and marbofloxacin) were the most frequently used antimicrobial agents in the treatment of calf diarrhea. In the USA, the administration of fluoroquinolones in food-producing animals is prohibited by law because of concerns regarding facilitating the emergence of bacteria with multiple resistances (Constable, 2004). Constable (2004) does not recommend fecal bacterial culture and antibacterial susceptibility testing in calves with diarrhea, because the fecal populations do not accurately reflect small intestinal bacterial populations and breakpoints for susceptibility testing have not been validated yet for fecal cultures. Based on his findings the latter author recommends the usage of ß-lactam antibiotics in calf diarrhea. In the present study, 10 farms exclusively used non-steroidal anti-inflammatory drugs (NSAID) in the treatment of calf diarrhea. Todd et al. (2010) demonstrated that calves with neonatal calf diarrhea complex experience some of the sickness behaviors, including change in appetite and depressed growth, and meloxicam (NSAID) is an effective supportive therapy in neonatal calf diarrhea. A metaphylactic use of therapeutics was reported for cryptosporidiosis and coccidiosis on the farms participating in the present study.

Florfenicol and macrolides (tilmicosin and tulathromycin) are the therapeutics commonly reported for treatment of respiratory infections in calves on the study farms. In Germany, *Pasteurella multocida* and *Mannheimia haemolytica*, both bacterial agents involved in BRD in calves have been found susceptible for amoxicillin/clavulanic acid, cefazolin, ceftiofur, cephalothin, chloramphenicol, florfenicol, doxycycline, enrofloxacin, trimethoprim, and trimethoprim/sulfamethoxazole, but resistance of *Pasteurella multocida* with respect to spectinomycin and tetracyclines has been demonstrated in about 16% and 11% of isolates, respectively (GERMAP, 2008).

In the present study penicillin was the most frequently used antimicrobial drug to treat navel ill. Usage of penicillin is justified due to the fact that anaerobes have been

demonstrated to play a role in umbilical infections. Penicillin, amoxicillin, ceftiofur, and fluoroquinolones are frequently used in the treatment of arthritis of calves.

5.4 Mastitis and antimicrobial usage

In this study, the farmers report that on average 2.8% of cows have to be treated for mastitis per month. A percentage of 12% cows treated for mastitis per month was the maximum rate. Mastitis is the most common reason for the administration of antimicrobials to dairy cows (Mitchell et al., 1998; Sawant et al., 2005; Pol and Ruegg, 2007). In addition, most control programs for mastitis on dairy farms include culturing of milk samples and subsequent antibacterial treatment (Owens et al., 1997; Thomson et al., 2008). In a survey on antibiotic usage in dairy herds in Pennsylvania, clinical mastitis occurred on all farms. In the latter study 14% of the lactating cows were treated with antibiotics due to clinical mastitis per year (Sawant et al., 2005).

In the present study, beta-lactams (especially cefquinome (81.7%) and penicillin (40.0%)), lincosamides (23.3%), and fluoroquinolones (20.0%) were the antimicrobial drug classes that were most often used in the treatment of cows with mastitis. All these drugs are licensed for lactational treatment of mastitis. Among fixed drug combinations, cefalexin+kanamycin (13.3%), ampicillin+cloxacillin (10.0%), and amoxicillin+clavulanic acid (8.3%) have been mentioned to be the most commonly used antibacterials in the treatment of mastitis. In accordance with this, in a previous study in dairy cows in Brandenburg, Germany (Tenhagen et al., 2006), cephalosporins (cefoperazone and cefquinome) were used on 35%, oxacillin or cloxacillin were used on 17%, and penicillin was used on 13% of the farms. In contrast, only one farm (1.7%) in the present study used tetracyclines in the treatment of mastitis, with oxytetracycline being used on 6% of the farms in the previous study.

In the US, drugs including penicillin G, penicillin G/ novobiocin, amoxicillin, cloxacillin, cephapirin, erythromycin, hetacillin, novobiocin and pirlimycin are approved for intramammary application during the lactation period (Sawant et al., 2005). In the survey on antibiotic usage in dairy herds in Pennsylvania, the drugs preferentially used for treating clinical mastitis were cephapirin (49%), penicillin G procaine (18%), and ceftiofur (18%) (Sawant et al., 2005). Pol and Ruegg (2007) reported that cephapirin (90%) and pirlimycin (75%) were the most frequently used intramammary antimicrobial drugs for treatment of clinical mastitis on 20 conventional farms in Wisconsin.

In a survey on publications regarding mastitis therapy Ruegg (2010) found more than 300 peer reviewed prospective studies published since 1990, of which less than 35 compare specific treatments for mastitis during lactation. Her conclusion was that the most popular intra mammary treatments in mastitis vary among countries without any supportive evidence that treatments used in one region are superior to those in another region. Most often the designs of the studies that were analyzed in the latter survey did not meet the criteria of proper scientific work, either due to the fact that farmers changed treatments during the trials or due to the fact that documentation was missing or the fate of some cows included in the study could not be verified. The usage of antibacterials in clinical mastitis, however, should be based on the bacteria involved in the disease, which vary from farm to farm. The survey mentioned above demonstrated that the definition of "cure" varied substantially between the different studies (Ruegg, 2010). A study on indication-based usage of antimicrobials in cattle in Finland revealed that intra mammary treatment was used in 34% of clinical cases of mastitis, and the most commonly used intra mammary antimicrobial agents were ampicillin in combination with cloxacillin (36%) and cephalexin+streptomycin (26%) (Thomson et al., 2008). In addition, in the latter study cephalexin+streptomycin (35%) and ampicillin+cloxacillin (18%) were the most commonly used intra mammary preparations for treatment of subclinical mastitis. In Sweden and Norway, the use of antibiotics for mastitis treatment has been influenced by national policies and recommendations. The preference for using beta-lactams (procaine, benzyl penicillin, and combinations with dihydrostreptomycin) was based on the withdrawal period in these countries (Grave et al., 1999).

For dry cow therapy, a study of 201 dairy herds in the Netherlands found that 82.8% of farmers used dry cow treatment on all cows (Barkema et al., 1998). Similarly, in the present study 85% of the farms performed dry cow treatment on all cows, and on 15% of the farms cows that received dry cow therapy were selected on beforehand. In the present study the preferred antimicrobial drugs for dry cow therapy on farms were penethamate hydroiodide+ benethamine penicillin + framycetinsulfate (48.3%), cloxacillin (38.3%), and cefquinome (15.0%). In a previous study on dairy farms in Brandenburg, Germany, cloxacillin alone or in combination, and penicillin were most commonly used for dry cow therapy (Tenhagen et al., 2006).

In the US, benzathine cephapirin, benzathine cloxacillin, erythromycin, novobiocin, penicillin G, penicillin G and novobiocin, and penicillin G and streptomycin are

intramammary antimicrobial drugs approved for dry cow therapy (Sawant et al., 2005). In 20 conventional dairy farms in Wisconsin, intramammary antimicrobial treatments were used in all quarters of all cows on all conventional farms at dry-off, and the most commonly used for intramammary dry cow therapy were penicillin (90%), streptomycin (90%), and cephapirin (75%) (Pol and Ruegg, 2007). Moreover, either cloxacillin alone or combined with ampicillin (50%), or beta-lactams combined with aminoglycosides (43%) were used as intramammary dry cow treatments in Finland (Thomson et al., 2008). In human medicine, all MRSA strains are considered to be resistant to penicillins, cephalosporins, and other beta-lactam antibiotics (Lee, 2003; Catry et al., 2010), and there is evidence that the use of a variety of antimicrobials is a major risk factor for colonization and infection (Catry et al., 2010). In addition, hospital-acquired MRSA strains are frequently resistant to most common antimicrobials including tetracyclines, aminoglycosides, macrolides, and fluoroquinolones (Lee, 2003). In veterinary medicine, the wide spread of MRSA ST 398 in food producing animal is related to the use of antimicrobials, particularly tetracycline (Catry et al., 2010). Van Duijkeren et al. (2008) found the number of MRSA ST 398 colonized pigs in farms that use standard antimicrobial medication to be higher compared to farms with no such use of antimicrobials. In Korea, the routine use of beta-lactam antibiotics for intramammary dry cow treatment in dairy herds might be a risk factor for the selection of MRSA strains (Lee, 2003; Moon et al., 2007).

5.5 The management of mastitis in the study farms

Among the 60 dairy farms in the present study, 70%, 60%, and 45% of the farms send milk samples from cows with clinical mastitis, with abnormal secreta or with high SCC, respectively, for bacteriological testing. In addition, 13.3% of the farms sent milk samples from postpartum cows and 20% sent the milk from cows before drying-off. This indicates good compliance with the principles of prudent use of antimicrobials as described in the guidelines issue by the German veterinary chamber, at least with respect to the treatment of mastitis.

An effective mastitis control program includes rapid identification and treatment of clinical mastitis cases, whole herd antibiotic dry cow therapy, post milking teat disinfection, culling of chronically infected cows, and routine maintenance of milking machines (Natzke, 1981; Dodd, 1983; Smith, 1983; Oliver and Mitchell, 1984; Harmon, 1996). Dairy producers should identify the causative mastitis pathogens in their herd for developing an

effective control program. Milk microbiological culture is a useful tool for identification of the pathogens that cause mastitis, and culture of milk from individual cows remains the most effective strategy to identify causative mastitis pathogens (Ferguson et al., 2007).

All farms included in the present study reported that they treated cows with clinical signs of mastitis with antimicrobial agents. Most of the farmers treated the cows when they found abnormal milk. About 40% treated cows with high SCC. In Finland, 37% of veterinarians based the diagnosis of acute mastitis on clinical signs, and veterinarians used bacteriological examination to target the treatment in the majority of cases (73%) of subclinical mastitis (Thomson et al., 2008). In the USA, on many farms, detection, diagnosis and administration of treatment for mild and moderate cases of clinical mastitis are the responsibility of farm personnel and veterinarians are often consulted only when a case becomes life-threatening. It is very important for veterinarians to be involved in developing and evaluating treatment protocols for clinical mastitis. The ability to assess the results of treatment is often limited because of inadequate records (Hoe and Ruegg 2006). Recording of antimicrobial treatments in food animals is mandatory in Germany. More than half of the study farms (58.6 %) recorded mastitis treatments on paper (cards or book), one-fourth of the farms used software programs, and 15.5% used both software programs and cards or book records. Therefore, there is potential for good treatment decisions that can be based – besides the results of clinical and bacteriological examinations of the current case – on well documented treatment records. In 113 dairy herds in Pennsylvania, 50% of dairy farms surveyed maintained antibiotic treatment records, and only 21% had a written plan for treating sick animals (Sawant et al., 2005). Insufficient record keeping and poor knowledge about drug withdrawal periods among producers were important factors leading to drug residues in milk (Kaneene and Ahl, 1987).

Evidence based veterinary medicine (EBVM) is an application of the principles of evidence based medicine, used by physicians, to clinical decision making for animals receiving veterinary care (Ruegg, 2010). The application of concepts of EBVM to mastitis therapy has the potential to improve treatment protocols and results of better therapeutic outcomes (Ruegg, 2010).

With respect to the duration of therapy in this study, 69.1% of the farms treated the cows for 3 to 4 days, 16.4% treated the cows for 1 to 2 days, and 14.5% treated for more than 4 days. Nearly one-third of the farms in this study reported they treated the cow up to the moment that the milk has a normal appearance again. In a previous study in dairy herds

in Brandenburg, Germany, 45% of farmers treated cows 3 to 4 times per case of mastitis and 22% of farmers administered further treatments or increased the duration of treatments (Tenhagen et al., 2006). Although supported by a number of studies that reported increasing success of treatment when treatment is prolonged (Oliver et al., 2004), this practice, conflicts with the legal obligation to preferentially use a drug as laid down in the description given by the pharmaceutical company, which was the basis of the licensing decision. Exceptions from this "rule" should only be made when justified by the outcome of the clinical examination. In Finland, the prescribed duration of treatment ranged from 1 to 8 days, and the median duration was 4 days (Thomson et al., 2008). In general, duration of antibiotic treatment is kept as short as possible to minimize the economic losses associated with milk discarded. Discarded milk is the greatest proportion of expense associated with treatment of clinical mastitis (Ruegg, 2010).

Half of the study farms left the cows that had been treated for mastitis in the milking herds, 45% separated those animals, and 5% used both options. In a previous study in Brandenburg, Germany, 20% of farms did not separate sick and treated cows from the milking cows, and two farms kept those cows among the herd mates without marking them (Tenhagen et al., 2006). The use of antimicrobial agents on farms always poses the risk of the milk becoming tainted with antibiotic residues (Zwald et al., 2004; Sawant et al., 2005). When antibacterials are discovered in the milk delivered to the dairy factory, farmers are fined. In addition, possible implications might rise for human health through an increasing risk of antimicrobial residues in dairy products (McEwen et al., 1991; Ruegg and Tabone, 2000; Ruegg, 2005). Quality assurance programs on dairy farms demand the identification and or separation of cows receiving antibacterials in order to guarantee that milk that is delivered to the dairy factory will not be contaminated. Physically separating treated cows, marking them visibly, and milking them last in separate milking units are effective in preventing drug residues in milk (Sawant et al., 2005). In Brandenburg, only 0.03 % of bulk tank samples were found positive for antibacterials in 2009 and 2010 (Landeskontrollverband Brandenburg, 2010).

5.6 Methicillin-resistant *Staphylococcus aureus* (MRSA)

Staphylococcus aureus is one of the most important contagious mastitis pathogens, which is frequently isolated from dairy cows with clinical (Bradley et al., 2007; Olde Riekerink et al., 2008; Tenhagen et al., 2008) and subclinical mastitis (Tenhagen et al., 2006; Bradley et al.,

2007; Ferguson et al., 2007). MRSA has been previously cultured in dairy cattle in many studies. The study of milk samples from cows with mastitis from 153 dairy farms in Korea found 2.5% MRSA positive (Moon et al., 2007). Vicca et al. (2008) reported that the percentage of MRSA positive cows in dairy herds varied from 0 to 14.3% in 5 dairy herds (1 located in the Netherlands, 4 in Belgium), and nearly 10% of 118 farms in Belgium have an MRSA problem (Vanderhaeghen et al., 2010). In a study on MRSA in three dairy herds in southwest Germany, 5.1 – 16.7 % of dairy cows were found positive for MRSA, and on those farms all bulk tank milk samples were found MRSA positive (Spohr et al., 2011).

In the present study, the prevalence of MRSA from bulk tank milk in the 60 dairy farms was 6.7%. The results from earlier publications and the present study indicate that bulk tank milk samples may be suitable for estimating the prevalence of MRSA in dairy herds. Using the same bacteriological methodology a monitoring program in Germany estimated the prevalence of MRSA in bulk tank milk in dairy herds at 4.1 % (Tenhagen et al. 2011). In contrast, the study on MRSA in US bulk tank milk reported that 218 bulk tank milk samples (40.2%) were positively cultured for *S. aureus*, but none were positive for MRSA on the selective indicator medium CHROMagar MRSA (Virgin et al., 2009). However, in the present study 25 ml of the bulk tank milk sample were enriched in the enrichment media, Mueller Hinton broth with 6.5% NaCl and Tryptone Soy Broth containing 3.5 mg/L cefoxitin and 75 mg/L aztreonam. In comparison, in the study in US bulk tank milk, the samples were cultured using routine methods for detecting mastitis pathogens including *S. aureus*. The difference in methodology may have contributed to the different results. An increase in the sensitivity of detecting MRSA in milk samples using the enrichment methods has been described (Spohr et al. 2011).

Recently, a study on MRSA in three dairy herds in southwest Germany revealed that quarters harbouring MRSA had higher SCC than quarters harbouring MSSA or being negative on culture (Spohr et al., 2011). In the latter study, MRSA was also detected in nasal swabs (7/15) and vaginal swabs (2/15) of cows. Three calves that were fed with MRSA-positive milk carried MRSA in their mouth and on their muzzles. Feeding non-pasteurized or mastitis milk to calves bears the risk of spreading multiresistant bacteria among the offspring. Increased SCC underlines that MRSA has to be considered a clinical problem in dairy cows, which is unlike the situation in fattening pigs where MRSA is only occasionally associated with clinical disease (van Duijkeren et al., 2007; Meemken et al. 2010).

In this study, cefquinome (4th generation of cephalosporins), penicillin, lincosamide, and fluoroquinolone were commonly used antimicrobial agents to treat mastitis. Among drug combinations, cefalexin+kanamycin and ampicillin+cloxacillin were commonly used. In addition, the most commonly used antimicrobials for intramammary dry cow therapy were penicillin, cloxacillin, and cefquinome. The use of intramammary treatments including penicillin, cloxacillin , and cephalosporins in the treatment of mastitis and for dry cow therapy may have contributed to the emergence of MRSA in bulk tank milk on the farms included in the present study as all these drugs are in the list of those drugs against which livestock-associated MRSA are resistant to. However, cows or even humans being carriers of this agent could contribute to its spread on the farm. In addition, the prudent usage of antimicrobial agents in dairy herds and the continuous screening for resistant microorganisms should be more focused to control MRSA on dairy herds (Moon et al., 2007).

Recently, MRSA ST398 has been isolated from farmers, family members, veterinary doctors and students, and slaughterhouse staff with a much higher frequency than in the rest of the population (Voss et al., 2005; Wulf et al., 2006; Van Loo et al., 2007; Wulf et al., 2007; Graveland et al., 2010; Spohr et al., 2011). The study on MRSA ST398 in veal calf farming in the Netherlands found that MRSA prevalence was 28% of veal calves and 88% at farms level, and there was a direct association between animal and human carriage of MRSA ST398. The prevalence of MRSA in the Dutch community is estimated to be below 1% (Wulf et al., 2006) whereas the overall prevalence of MRSA was 15.9% in persons living and working on a veal calf farm (Graveland et al., 2010). In addition, MRSA prevalence in farmers was 33% and 8% in family members, with some hints that the time spent inside the stables could have contributed to the carrier status. In the study in the Netherlands, MRSA ST398 carriage in healthcare personnel in contact with pigs and veal calves was 1.7% and in the control group was 0.15%, and the result from this study demonstrated that MRSA ST398 carriage in healthcare personnel in contact with farm animals is 10-fold higher than in other healthcare personnel (Wulf et al., 2008). In the study on MRSA on three dairy herds in southwest Germany, MRSA were detected in nasal swabs of staff members (7/9), cows (7/15), and calves (4/7), and bulk tank milk (3/3). Moreover, this MRSA ST398 type has also been isolated from human patients (Krziwanek et al., 2009; van Cleef et al., 2011). Detection of MRSA in veal calves, in dairy cows with clinical or subclinical mastitis, in bulk tank milk, in dairy calves, in staff working on the farm, in

farmers and in family members confirms the ability of MRSA to be transmitted from animals to humans or vice versa.

In the present study on 36 MRSA isolates from bovine milk samples, two different *spa*-types were identified, including t011 and t034, and the dominant type was t011 (n = 22). In Germany, *spa*-types from many previous studies of MRSA in pigs (Kadlec et al., 2009; Tenhagen et al. 2009), in dairy cows (Feßler et al., 2010; Spohr et al., 2011), and in veal calves and humans working or living on farms in the Netherlands (Graveland et al., 2010) have been reported. Similarly to the present study, t011 and t034 were the predominant *spa*-types. Moreover, a study of MRSA ST398 in cases of clinical and subclinical mastitis in Belgian cows had similar results (Vanderhaeghen et al., 2010).

For SCC*mec* typing in this study, 33 strains had SCC*mec* type V, 2 strains had SCC*mec* type III, and only one strain had SCC*mec* type IVa. This finding is in agreement with the recent study of MRSA ST398 from cases of bovine mastitis in Germany, the dominant SCC*mec* type was type V (Feßler et al., 2010), and the bulk tank milk sample in three dairy herds in southwest Germany belonged to SCC*mec* type V (Spohr et al., 2011). In the previous study on 54 MRSA ST398 isolates from unrelated diseased swine collected all over Germany, shows a similar result that 53 MRSA isolates were SCC*mec* type V (Kadlec et al., 2009). Additionally, in the present study most of the *spa*-type t011 isolates belonged to the SCC*mec*-type V (95.5%) and the 2 strains that have SCC*mec*-type III belonged to *spa*-type t034. This finding is in agreement with the study of MRSA in slaughter pigs in Germany that 92% of SCC*mec*-type V isolates belonged to *spa*-type t011, and 94% of SCC*mec*-type III isolates belonged to *spa*-type t034 (Tenhagen et al., 2009). The widespread existence of a homogenous group of MRSA indicates clonal spread of this strain within the dairy cow populations in Germany.

5.7 Phenotypic and genotypic antimicrobial resistance of MRSA

All strains isolated from milk in this study were found resistant to oxacillin (OXA) and tetracyclines (TET). In Korea, 90.5% of MRSA positive strains isolated from dairy cows were found resistant to penicillin and ampicillin, but only 23.8% were resistant to tetracyclines (Moon et al., 2007). In the study of MRSA ST398, isolated from clinical and subclinical mastitis in Belgian cows all were resistant to tetracyclines (Vanderhaeghen et al., 2010), and all strains of MRSA ST 398 isolated from diseased swine in Germany were resistant to beta-lactams and tetracyclines (Kadlec et al., 2009; Tenhagen et al. 2009).

From phenotypic antimicrobial susceptibility testing in this study, 16 resistance patterns were detected, and the most common resistance pattern was TET-OXA alone which was found in 10 strains (27.8%). A recent study on MRSA ST398 from cases of bovine mastitis in Germany, revealed the presence of 10 different susceptibility patterns, and the most frequently seen resistance pattern was resistance to beta-lactams and tetracyclines only. It was detected in 11 of 25 isolates (Feßler et al., 2010).

In addition, all 36 MRSA strains isolated from bovine milk in this study were susceptible to mupirocin, vancomycin, and linezolid. In Germany, in studies on MRSA ST398 from diseased swine (Kadlec et al., 2009) and from cases of bovine mastitis (Feßler et al., 2010), all isolates were susceptible to vancomycin, with a MICs range of 0.5-1 mg/L. In Switzerland, all strains of MRSA isolated from livestock and veterinarians were also susceptible to vancomycin (Huber et al., 2010).

The DNA microarray analysis results obtained in this study showed that all 36 MRSA ST398 strains isolated from bovine milk harboured the gene encoding resistance to methicillin, oxacillin, and all beta-lactams (*mec*A) and 35 strains (97.2%) harboured *bla*I, *blaZ*, *bla*R (beta-lactamase). Recently, studies on 25 MRSA ST398 strains isolated from cases of bovine mastitis in Germany (Feßler et al., 2010) and on 20 MRSA strains isolated from livestock and veterinarians in Switzerland (Huber et al., 2010) showed similar results that all strains harboured the *mec*A gene and genes encoding the region responsible for the synthesis of beta-lactamase (*bla*I, *blaZ*, *bla*R). In the present study, all strains showed phenotypic resistance to tetracycline which was based on the presence of either two *tet*M +*tet*Efflux (17 isolates) or three *tet*M+*tet*Efflux+*tet*K (19 isolates) resistance genes. As previously seen in MRSA ST398 isolated from cases of bovine mastitis in Germany, the most common genes encoding for tetracycline resistance were *tet*M, *tet*K, and *tet*L (Feßler et al., 2010), and all 20 MRSA strains isolated from livestock and veterinarians in Switzerland harboured gene *tet*M (Huber et al., 2010). In addition, among 54 tetracycline-resistant isolates of MRSA ST398 from diseased swine all over Germany, 40 carried the genes *tet*M+*tet*K, 11 *tet*M+*tet*K+*tet*L, and one isolate revealed the presence of *tet*M+*tet*L, *tet*M or *tet*L (Kadlec et al., 2009). Recently, all of the ST398 isolated from different sources (healthy carrier and diseased pigs, dust from pig farms, milk, and meat) in Germany showed resistance to tetracycline encoded by *tet*M alone or together with *tet*K and/or *tet*L (Argudin et al., 2011).

In the present study, genes for vancomycin resistance (*van*A, *van*B, *van*Z) were found in none of the 36 MRSA strains isolated from bovine milk, and this is in accordance with a study on MRSA strains isolated from livestock and veterinarians in Switzerland (Huber et al., 2010). Vancomycin is an antibiotic commonly used in human medicine for treatment of infections caused by MRSA (Micek, 2007).

In this study, 21 strains showed combined phenotypic resistance to erythromycin and clindamycin. In 18 strains the genes for marcolide and lincosamide resistance (*erm*A, *erm*B, *erm*C) were detected. It should be noted that the resistance genes *erm*A, *erm*B, *erm*C have also been detected in MRSA ST398 from cases of bovine mastitis (Feßler et al., 2010), from diseased swine (Kadlec et al., 2009), and from other sources (Argudin et al., 2011) in Germany. Only one isolate (2.8%) of MRSA from a milk sample in this study carried *fex*A gene (chloramphenicol) and also exhibited a high MIC value of 64 µg/ml for chloramphenicol. In accordance with this study, in a study on 54 MRSA ST398 isolates from diseased swine, two chloramphenicol-resistant isolates (MICs 64-128 µg/ml) were detected and both carried the gene encoding for chloramphenicol resistance (Kadlec et al., 2009), and two of 25 MRSA ST398 from cases of bovine mastitis harboured the *fex*A gene (Feßler et al., 2010).

None of the strains in this study obtained the gene encoding resistance to trimethoprim (*dfr*A) while 5 strains showed phenotypic resistance to trimethoprim/sulfamethoxazole. In contrast, trimethoprim resistance was found in 28 MRSA ST398 strains isolated from diseased swine with 14 of them carrying resistance gene *dfr*K (Kadlec et al., 2009), and the gene encoding resistance to trimethoprim (*dfr*A) was detected in three human strains from 20 MRSA strains isolated from livestock and veterinarians in Switzerland (Huber et al., 2010). Recently, in 12 out of 25 MRSA ST398 strains isolated from cases of mastitis, a gene encoding resistance to trimethoprim (*dfr*K) was detected (Feßler et al., 2010). However, this gene was not included in the test procedure in this study and may have been responsible for the observed phenotypic resistance.

For 13 MRSA strains that showed phenotypic resistance to streptogramin (quinupristin/dalfopristin), 8 strains harbored *erm*A, 1 strain harbored *erm*C or *erm*A and *erm*B or *erm*A and *erm*C, and 2 strains harbored *erm*C and *vga*A. Similarly, a study in bovine mastitis MRSA ST398 isolates showed that resistance genes *erm*A, *erm*B, and *erm*C were detected alone or in different combinations (Feßler et al., 2010). Moreover, the genes for aminoglycoside resistance (*aac*A-*aph*D, *aad*D, *aph*A) were detected in 7 strains from 10

strains that showed phenotypic resistance to kanamycin. The aminoglycoside resistance genes *aad*D, *aac*A-*aph*D have also been identified in porcine MRSA ST398 isolates (Kadlec et al., 2009) and in bovine mastitis MRSA ST398 isolates (Feßler et al., 2010).

5.8 Genotypic study for enterotoxins, toxic shock syndrome toxins, leukocidins, and hemolysins of MRSA

In the present study, all 36 MRSA ST398 strains isolated from bovine milk were staphylococcal enterotoxins and PVL negative. This finding is in accordance with the study of MRSA ST398 from cases of bovine mastitis (Feßler et al., 2010). In a previous study on diseased swine in Germany, all 54 MRSA ST398 isolates were PVL negative, but one isolate was positive for enterotoxin B gene (*seb*) and another three isolates were positive for enterotoxin K and Q genes (*sek* and *seq*) (Kadlec et al., 2009). In Switzerland, one strain isolated from veterinarian harboured 4 enetrotoxin genes (*ent*A, *ent*B, *ent*K, *ent*Q) and one strain isolated from cattle harboured enterotoxin gene *ent*H (Huber et al., 2010).

For the genes encoding leukocidins/haemolysin toxin family protein, the DNA microarray analysis showed *luk*X and *luk*Y-var1 to be present in all strains in this study. In contrast, the study on 20 MRSA strains isolated from livestock and veterinarians in Switzerland revealed that in almost every strain genes encoding leukocidins, *luk*F, *luk*S, *luk*Y were detected (Huber et al., 2010). In addition, all 36 MRSA ST398 strains harbored *hla* (haemolysin alpha), un-truncated *hlb* (haemolysin beta), *hld* (haemolysin delta), *hl* (hypothetical protein similar to haemolysin), *hl*III, *hl*_III_other than RF122 (putative haemolysin III), and *luk*F, *luk*S, *hlg*A (haemolysin gamma). The genes encoding haemolysin alpha (*hla*), delta (*hld*), and gamma (*hlg*A) have also been identified in MRSA strains isolated from livestock and veterinarians (Huber et al., 2010, Argudin et al. 2011).

5.9 Extended-spectrum beta-lactamases producing *E. coli*

In the present study, 30% of the farms found ESBLs producing *E. coli* in their bulk tank milk. The use of antimicrobials for treatment of cows with dystocia and frequent occurrence of metritis and dystocia were associated with the detection of ESBLs-producing *E. coli* in bulk tank milk. This study also demonstrated that the occurrence of dystocia was positively correlated to the occurrence of metritis and the use of antimicrobials for treatment of dystocia. This finding indicated that cows with dystocia are more likely to develop metritis, which consequently leads to the administration of antimicrobials on farms which

subsequently poses the risk to the emergence of ESBLs-producing *E. coli* in the farms. The use of ceftiofur, a third generation cephalosporin, for the treatment of metritis has repeatedly been recommended (Drillich et al., 2006). In line with this, ceftiofur was frequently used for the treatment of metritis in this study. This underlines that farmers and veterinarians should be aware not only about the prudent use of antimicrobials but also about the controll of diseases or health problems on their farms.

The practice of adding antimicrobials to feed has the potential to contribute to the emergence of antimicrobial-resistant commensals and/or pathogenic bacteria. Berge et al. (2006) carried out a field trial evaluating the effect of prophylactic and therapeutic antimicrobial administration on antimicrobial resistance of fecal *E. coli* in dairy calves. Results revealed that calves that fed neomycin and tetracycline in the milk replacer had higher levels of multiple antimicrobial resistance in fecal *E. coli.* Individual antimicrobial therapy also increased resistance in bacteria isolated from calves but this increase appeared to be transient. Moreover, a study of Alexander et al. (2008) in feedlot cattle indicated that subtherapeutic administration of tetracycline in combination with sulfamethazine increased the prevalence of tetracycline- and ampicillin-resistant *E. coli*.

In German hospitals in the last 10 to 15 years, increasing rates of multidrug resistant *E. coli* have been recorded. The increase in resistance against third and fourth generation cephalosporins can be explained in part by the emergence of ESBLs -producing organisms (GERMAP, 2008). The recommendations of the joint FAO/WHO/OIE expert meeting (FAO/WHO/OIE, 2007), come to the conclusion that there is an overlap for 3rd and 4th generation cephalosporins, quinolones, macrolides, penicillins and aminoglycosides in the list of critically important antimicrobials for human health and animal health. This overlap highlights the need for antimicrobial resistance surveillance and to identify and implement appropriate management measures in order to mitigate resistance dissemination and maintain the efficacy of the drugs. Prudent use of all antimicrobials is considered essential. Foodborne pathogens and commensals (in particular *Salmonella* spp., *Campylobacter* spp. and *E. coli*) linked to potential antimicrobial resistance to 3rd and 4th generation cephalosporins, quinolones and macrolides should be given special consideration for risk analysis.

5.10 Antimicrobial resistance of ESBLs-producing *E. coli*

All strains isolated from bulk tank milk in this study were found resistant to ampicillin, cephalothin, ticarcillin, piperacillin, ceftiofur, and cefpodoxime. Resistance to ciprofloxacin (27.8%), nalidixic acid (33.3%), tetracycline (38.9%), streptomycin (27.8%), and sulfonamide (38.9%) was detected in high frequency. Resistance to chloramphenicol was found in 11.1% of isolates, but all strains were susceptible to florfenicol and spectinomycin. In comparison, the study on ceftiofur-resistant *E. coli* isolated from the feces of healthy dairy calves showed that the isolates were resistant to ampicillin (100%), ceftiofur (100%), chloramphenicol (94%), florfenicol (93%), spectinomycin (72%), and tetracycline (98%) (Donaldson et al., 2006). In contrast to calves, florfenicol is not licensed for use in dairy cows in Germany. The latter study revealed that healthy dairy calves were rapidly colonized by antibiotic-resistant strains of *E. coli* shortly after birth, and multidrug resistant nonpathogenic *E. coli* in calves, which could from a significant source of resistance genes to other bacteria that share the same environment.

5.11 Bulk tank milk may be the source of MRSA and ESBLs-producing *E. coli*

Milk quality continues to be a topic of vital interest to the dairy industry and in the public health communities. High quality milk contains a low number of somatic cells, a low bacterial count, and is free of human pathogens and antibiotic residues (Oliver et al., 2009). Currently, consumers from all over the world are demanding dairy products that are nutritious, safe, and produced from healthy cows. Production of high-quality milk is an important goal of every dairy producer. Moreover, the number of people consuming raw milk in the United States is increasing, because of the belief that raw milk can enhance nutritional quality, taste, and health benefits (Oliver et al., 2009).

Bulk tank milk has been used to monitor udder health and milk quality in a dairy herd. A study from Jayarao et al. (2004) revealed that an increase in the frequency of isolation of *S. aureus* and *Streptococcus agalactiae* was significantly associated with an increase bulk tank somatic cell count, and bulk tank milk with low standard plate counts also had a significantly lower level of mean bulk tank somatic cell count. Oliver et al. (2005) recently reviewed the prevalence of milkborne pathogens from bulk tank milk and farm environments. The routine monitoring of bacteria obtained from bulk tank milk may be an important tool for detecting the trends in antimicrobial resistance on dairy farms. In the study in California, USA, 23% of all isolates (*E. coli* and Salmonella) from bulk tank milk

were classified as multidrug resistant (Berge et al., 2007). *E. coli* in bulk tank milk is likely a result of fecal contamination (Jayarao and Wang, 1999).

The conclusion remarks with respect to the findings of the present study are as follows:

A questionnaire addressing herd managers of 60 dairy demonstrated that lameness and metritis were considered the predominant disorders in adult dairy cows. Ceftiofur was reported to be most frequently used in the treatment of lame cows, which originates from the fact that it is registered for digital phlegmona and has no withdrawal time with respect to milk. Amoxicillin, ceftiofur, and tetracyclines were preferentially used in the treatment of metritis. In calves, respiratory disease and calf diarrhea were mentioned to be the most important diseases on the dairy farms participating in the present study. Fluoroquinolones were the most frequently used antimicrobial agents to treat calf diarrhea, whereas florfenicol and macrolides were commonly used in the treatment of respiratory disease.

In this study, the farmers reported that treatment of mastitis per month was 2.8% of cows on average, and cefquinome, a 4th generation cephalosporin, was the most frequently used antimicrobial agent. Dry cow therapy included the entire herd on most of the farms. The preferential antimicrobial drugs for dry cow therapy were penethamate hydroiodide+ benethamine penicillin + framycetinsulfate, cloxacillin, and cefquinome.

The important criteria for sending milk samples for bacteriological examination to the laboratory were cows with clinical mastitis, abnormal secreta, and high SCC. All the farms in this study reported that they treated the cows with signs of clinical mastitis with antimicrobial agents, and most of the farms treated the cows for 3 to 4 days. Half of the study farms left the cows that had been treated for mastitis in the group, and 58.6% of the study farms recorded mastitis treatments on paper.

In the present study of 36 MRSA isolated from bovine milk samples, only two different *spa*-types were identified, including t011 and t034. Considering SCC*mec* typing, 33 strains had SCC*mec* type V, 2 strains had SCCmec type III, and only one strain had SCCmec type IVa.

From phenotypic antimicrobial susceptibility testing in this study, all MRSA strains isolated from milk were resistant to oxacillin and tetracycline, whereas none of the strains isolated from milk were resistant to vancomycin, mupirocin or linezolid.

The DNA microarray analysis results obtained in this study showed that all 36 MRSA ST398 strains isolated from bovine milk harboured the gene encoding resistance to methicillin, oxacillin, and all beta-lactams (*mec*A), and all strains were staphylococcal enterotoxins and PVL negative.

The use of antimicrobials for treatment of cows with dystocia and frequent occurrence of metritis and dystocia were associated with the detection of ESBLs-producing *E. coli* in bulk tank milk. All ESBLs-producing *E. coli* strains were found resistant to ampicillin, cephalothin, ticarcillin, piperacillin, ceftiofur, and cefpodoxime.

In the present study, 6.7% of bulk tank milk samples were found MRSA positive, and on 30% of the farms ESBLs-producing *E. coli* were detected in bulk tank milk at a single sampling. This underlines the need to heat treat milk before marketing and consumption. MRSA and ESBLs-producing *E. coli* in bulk tank milk in this study were detected using enrichment methods, therefore the amount of MRSA and ESBLs-producing *E. coli* was not determined. The result in this study indicate that bulk tank milk could be the source of MRSA and ESBLs-producing *E. coli*, and that testing bulk tank milk samples might be a suitable tool for monitoring the presence of multiresistent bacteria in animal products that are used for human consumption.

Further activities with respect to a reduction of multiresistent bacteria in the food animal sector should focus on more transparency in the usage of antibacterials in food producing animals e.g. by establishing farm specific treatment protocols for the most common disorders in which 3rd and 4th generation cephalosporins and fluoroquinolones are mostly omitted as well as establishing a surveillance system including a central register where antibiotic treatments have to be reported. Financial incentives should be provided for farmers that demonstrate a consistently low usage of antimicrobials and manage to keep their cattle healthy at the same time.

Chapter 6

Summary

Usage of Antimicrobials on 60 Dairy Farms in Northern Germany and Characterization of Methicillin-Resistant *Staphylococcus aureus* (MRSA) and Extended Spectrum Beta-Lactamases Producing *Escherichia coli* (ESBLs-producing *E. coli*) Isolated from Bulk Tank Milk Samples.

The objectives of this study were to gain insight into the usage of antimicrobials on dairy farms in Germany and into the presence of MRSA and ESBLs-producing *E. coli* in bulk tank milk samples. To this end a questionnaire was performed among herd managers of 60 farms (herd size from 25 to 3,000 animals) in Northern Germany, who were participating in the study on a voluntary basis. Bulk tank milk samples were obtained at a single occasion and analysed for the presence of MRSA and ESBLs-producing *E. coli*. In addition MRSA isolates from milk from the strain collection of the National Reference Laboratory for coagulase positive staphylococci incl. *S. aureus* (NRL Staph) were characterized by multiplex PCR, SCC*mec* typing, *spa* typing, and DNA micro array analysis.

Herd managers reported that lameness, metritis and mastitis were regarded as the most important health problems of cows. Neonatal calf diarrhea and the bovine respiratory disease complex (BRD) were reported as the most important disorders in calves. Beta-lactams, tetracyclines, macrolides, sulfonamides, fluoroquinolones, aminoglycosides, phenicols, and polypeptides were the antimicrobial drug classes that were administered to diseased animals on the dairy farms. A third generation cephalosporin – ceftiofur – which is registered for use in digital phlegmona was the antimicrobial most frequently administered to lame cows. Amoxicillin, tetracyclines and ceftiofur were preferentially used in the treatment of metritis. BRD in calves was mostly treated with florfenicol or macrolides, whereas fluoroquinolones were most frequently administered to diarrheic calves.

The herd managers reported that on average 2.8% of their cows were treated for clinical mastitis per month. Milk samples were sent for bacteriological examination in case of clinical mastitis, or, less frequently, from cows with elevated somatic cell counts (SCC). The most frequently mentioned antimicrobial agents that have been used in the treatment of

cows with mastitis were cefquinome, penicillin, and the fixed drug combination cefalexin+kanamycin and ampicillin+cloxacillin.

On the majority of farms (85% of the farms) a routine procedure at drying off is applied, which includes the intra mammary administration of antimicrobials. Cloxacillin and cefquinome and the fixed drug combination penethamate hydriodide + benethamine penicillin + framycetinsulfate were the antimicrobial drugs mainly used for dry cow therapy.

In total 36 MRSA isolates from bovine milk were characterized. Five isolates originated from the bulk tank samples obtained during the farm visits and 31 isolates originated from the strain collection of the NRL Staph. All isolates were confirmed to be MRSA by multiplex PCR and DNA micro array analysis. Two different *spa* types were identified, namely t011 (22 isolates) and t034 (14 isolates). Among those, 33 carried SCC*mec* type V, 2 strains had SCC*mec* type III, and only one strain had SCC*mec* type IVa.

All MRSA strains were phenotypically resistant to oxacillin and tetracyclines. None of the strains was resistant to mupirocin, vancomycin, or linezolid. In total 16 resistance patterns were detected, and the most common resistance pattern was TET-OXA alone. Multidrug resistance (MDR) to a range of three to seven antimicrobial agents was found in 72.2% of the isolates. The most predominant multidrug resistance pattern was TET-ERY-CLI-OXA-QUI/DAL.

All MRSA strains carried more than one antimicrobial resistance gene, and 18 different antimicrobial resistance gene patterns were identified. The most common genotypic resistance patterns were *mec*A-*bla*Z-*bla*I-*bla*R-*tet*M-*tet*Efflux-*tet*K and *mec*A-*bla*Z-*bla*I-*bla*R-*tet*M-*tet*Efflux-*erm*A. All 36 MRSA strains carried the genes *mec*A, *tet*M, and *tet*Efflux, and tested negative for *van*A, *van*B, *van*Z (vancomycin), *msr*A, *mef*A, *mpb*BM (macrolides), *lin*A, *cfr* (lincosamides), *vat*A, *vat*B, *vga*, *vgb* (streptogramin), *aph*A (aminoglycoside), *dfr*A (trimethoprim), and *cat* (chloramphenicol).

In the present study, all strains were tested negative for genes encoding for enterotoxins, genes encoding for toxic shock syndrome toxins (*tst*1, *tst*-RF122), Pantone-Valentine Leukocidin (PVL), leukocidins (*luk*M/*luk*F-P83, *luk*D, *luk*E, *luk*Y-var2), and hemolysin Beta (*hlb*). In addition, all strains harbored *luk*X and *luk*Y-var1 (leukocidins/haemolysin toxin family protein), *hla* (haemolysin alpha), un-truncated *hlb* (haemolysin beta), *hld* (haemolysin delta), and *luk*F, *luk*S, *hlg*A (haemolysin gamma).

ESBLs-producing *E. coli* were isolated from bulk tank milk samples from 30% of the farms. Detection of ESBLs-producing *E. coli* was associated with the occurrence of metritis and dystocia on the farms. The use of antimicrobials in the treatment of cows following dystocia and in the treatment of umbilical infections and arthritis in calves was also associated with the presence of ESBLs-producing *E. coli* in bulk tank milk. All ESBLs-producing *E. coli* strains were resistant to ampicillin, cephalothin, ticarcillin, piperacillin, ceftiofur, and cefpodoxime. None of the strains were found resistant to florfenicol, spectinomycin, amoxicillin + clavulanic acid, ceftazidime, and cefoxitin.

In conclusion, the results of the questionnaire demonstrate that fluoroquinolones, oxacillin and cloxacillin as well as 3rd and 4th generation cephalosporins are administered to milking cows and their offspring as initial treatment for the most frequently occurring diseases on dairy farms. Testing bulk tank milk seems a suitable tool to monitor the presence of MRSA and ESBLs-producing *E. coli* in milk. The presence of the latter bacteria in milk underlines the need to heat treat milk before consumption.

Chapter 7

Zusammenfassung

Einsatz von Antibiotika in 60 Milchrinderbetrieben in Norddeutschland und Charakterisierung von Methicillin-resistenten *Staphylococcus aureus* (MRSA) und Extended-Spectrum Beta-Lactamase produzierenden *Escherichia coli* (ESBL-produzierende *E. coli*)

Das Ziel der Studie war, Informationen über den Einsatz von Antibiotika in deutschen Milchkuhherden zu sammeln. Zudem sollte auf das Vorkommen von MRSA und ESBL-produzierenden *E. coli* in Tankmilchproben untersucht werden. Fragebögen wurden unter den Herdenmanagern von 60 norddeutschen Betrieben (Herdengröße von 25 bis 3000 Tiere) verteilt, die auf freiwilliger Basis an den Untersuchungen teilnahmen. Tankmilchproben wurden in den Betrieben einmalig entnommen und auf das Vorkommen von MRSA und ESBL-produzierenden *E. coli* untersucht. Zusätzlich wurden MRSA-Isolate aus Milch aus der Stammsammlung des Nationalen Referenzlabors für koagulase-positive Staphylokokken (inklusive *S. aureus*) (NRL Staph) mittels Multiplex PCR, Scc*mec*-Typisierung, *spa*-Typisierung und einem DNA-Microarray analysiert.

Die Herdenmanager benannten Lahmheiten, Metritiden und Mastitiden als häufigste Krankheitsprobleme der Kühe ihrer Betriebe. Neonataler Kälberdurchfall und der Kälbergrippekomplex (BRD) wurden als Hauptprobleme bei Kälbern angegeben. Beta-Lactame, Tetrazykline, Makrolide, Sulfonamide, Fluoroquinolone, Aminoglykoside, Phenikole und Polypeptide waren die für erkrankte Tiere eingesetzten Antibiotikaklassen auf den Betrieben. Ein Cephalosporin der dritten Generation – Ceftiofur –, das zur Behandlung der Unterfußphlegmone (Panaritium) zugelassen ist, war das zur Behandlung von Lahmheiten der Kühe meist genutzte Antibiotikum. Amoxicillin, Tetrazykline und Ceftiofur wurden bevorzugt zur Behandlung von Metritiden eingesetzt. BRD bei Kälbern wurde zumeist mit Florfenicol or Makroliden behandelt, während Fluoroquinolone die Mittel der Wahl bei Durchfallkälbern waren.

Nach Angaben der Herdenmanager wurden monatlich im Durchschnitt 2.5% der Kühe aufgrund einer klinischen Mastitis behandelt. In solchen Fällen wurden routinemässig Milchproben zur bakteriologischen Untersuchung eingeschickt, seltener Proben von Kühen

mit erhöhten somatischen Zellzahlen. Für die Behandlung von Mastitiden wurden Cefquinom, Penizillin und die Kombinationen Cefalexin+Kanamycin und Ampicillin+Cloxacillin als häufigste genutzte Antibiotika angegeben.

Die meisten der untersuchten Betriebe (85% der Betriebe) wenden Routineverfahren zum Trockenstellen an. Dies beeinhaltet eine intramammäre Applikation von Antibiotika. Cloxacillin und Cefquinom und die Kombination von Penethamathydrojodid + Benethamin-Penizillin + Framycetinsulfat wurden am häufigsten zum Trockenstellen eingesetzt.

Insgesamt wurden 36 MRSA-Isolate aus Kuhmilch charakterisiert. Fünf Isolate wurden dabei aus Tankmilchproben, die während der Betriebsbesichtigungen genommen wurden, gewonnen. 31 Isolate stammten aus der Stammsammlung des NRL Staph. Die Zuordnung der Isolate zu MRSA wurde Mithilfe einer Multiplex PCR und DNA-Microarrayanalysen verifiziert. Zwei verschiedene *spa*-Typen konnten ermittelt werden: t011 (22 Isolate) und t034 (14 Isolate). Dabei konnten 33 Stämme dem SCC*mec* Typ V, 2 Stämme dem SCC*mec* Typ III und nur ein Stamm dem SCC*mec* Typ IVa zugeordnet werden.

Alle MRSA-Stämme zeigten im Phänotyp Resistenzen gegenüber Oxacillin und Tetrazyklinen. Kein Stamm zeigte Resistenzen gegenüber Mupirocin, Vancomycin oder Linezolid. Insgesamt wurden 16 Resistenzmuster detektiert. Das häufigste Resistenzmuster war TET-OXA. Mehrfachresistenzen (multidrug resistance-MDR) gegenüber drei bis sieben Antibiotika konnten in 72,2% der Isolate nachgewiesen werden. Das häufigste Mehrfachresistenzmuster war dabei TET-ERY-CLI-OXA-QUI/DAL.

Alle MRSA-Stämme trugen mehr als ein Antibiotikaresistenzgen. 18 verschiedene Antibiotikaresistenz-Genmuster wurden identifiziert. Die häufigsten Antibiotikaresistenz-Genmuster waren *mec*A-*bla*Z-*bla*I-*bla*R-*tet*M-*tet*Efflux-*tet*K und *mec*A-*bla*Z-*bla*I-*bla*R-*tet*M-*tet*Efflux-*erm*A. Alle 36 MRSA trugen die Gene *mec*A, *tet*M und *tet*Efflux, jedoch konnten keine *van*A, *van*B, *van*Z (Vancomycin), *msr*A, *mef*A, *mpb*BM (Macrolide), *lin*A, *cfr* (Lincosamide), *vat*A, *vat*B, *vga*, *vgb* (Streptogramin), *aph*A (Aminoglycoside), *dfr*A (Trimethoprim) und *cat* (Chloramphenicol) nachgewiesen werden.

In der vorgelegten Studie konnten in keinem Stamm Enterotoxin-Gene bzw. Gene, die toxische-Schock-Syndrom-Toxine (*tst*1, *tst*-RF122), Panton-Valentin Leukozidin (PVL), Leukozidine (*luk*M/*luk*F-P83, *luk*D, *luk*E, *luk*Y-var2), und Hämolysin Beta (hlb) kodieren,

nachgewiesen werden. Alle Stämmen trugen *luk*X und *luk*Y-var1 (Leukozidine/haemolysin toxin family protein), *hla* (Hämolysin Alpha), un-truncated *hlb* (Hämolysin Beta), *hld* (Hämolysin Delta) und *luk*F, *luk*S, *hlg*A (Hämolysin Gamma).

ESBL-produzierende *E. coli* wurden in Tankmilchproben von 30% der Betriebe nachgewiesen. Der Nachweis von ESBL-produzierenden *E. coli* ging mit dem Auftreten von Metritis und Dystokie in den entsprechenden Betrieben einher. Der Einsatz von Antibiotika nach Schwergeburten sowie die Behandlung von Kälbern mit Nabelinfektionen und Arthritis konnten mit dem Auftreten von ESBL-produzierenden *E. coli* in Tankmilch assoziiert werden. Alle ESBL-produzierenden *E. coli* waren resistent gegenüber Ampicillin, Cephalothin, Ticarcillin, Piperacillin, Ceftiofur und Cefpodoxim. Kein Stamm zeigte Resistenzen gegenüber Florfenicol, Spectinomycin, Amoxicillin + Clavulansäure, Ceftazidim und Cefoxitin.

Zusammengefasst zeigen die Daten der Fragebögen, dass Fluorchinolone, Oxacillin, Cloxacillin und Cephalosporine der 3. und 4. Generation zur initialen Behandlung der häufigsten Erkrankungen bei Milchkühen und Kälbern eingesetzt werden. Die Untersuchung von Tankmilch ist ein geeignetes Verfahren zur Überprüfung des Vorkommens von MRSA und ESBL-produzierenden *E. coli* in Milch. Das Vorkommen von ESBL-produzierenden *E. coli* in Tankmilch unterstreicht die Notwendigkeit, Milch vor dem Verzehr zu erhitzen.

Chapter 8
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Chapter 9

Appendix

A. Abbreviations

A	
AABP	American Association of Bovine Practitioners
AA-MRSA	Animal-Associated Methicillin-resistant *Staphylococcus aureus*
AMC	amoxicillin with clavulanic acid
AMDUCA	Animal Medicinal Drug Use Clarification Act
AMP	ampicillin
ATCC	American Type Culture Collection
AVMA	American Veterinary Medical Association
AZM	Aztreonam
B	
BAP	blood agar plates
BfR	Bundesinstitut für Risikobewertung or Federal Institute for risk assessment
BHI	Brain heart infusion
BRD	Bovine Respiratory Disease complex
BTK	Bundestierärztekammer or Germany's National Veterinary Association
C	
ºC	Degree Celcius
CA-MRSA	Community-Associated Methicillin-resistant *Staphylococcus aureus*
CAZ	ceftazidime
CEF	cephalothin
CHL	chloramphenicol
CIP	ciprofloxacin
CLI	clidamycin
CLSI	The Clinical and Laboratory Standard Institute
CNS	Coagulase-negative staphylococci
CPD	cefpodoxime
CRO	ceftriaxone
CTX	cefotaxime
CXM	cefuroxime
D	
d	day
DDD	defined daily doses
DNA	Deoxyribonucleic acid
E	
EARS-Net	European Antimicrobial Resistance Surveillance Network
ECDC	European Centre for Disease Prevention and Control
E. coli	*Escherichia coli*
EFSA	The European Food Safety Authority
EFT	ceftiofur
EMEA	The European Agency for the Evaluation of Medicinal Products

ERY	erythromycin
ESBLs	Extended-spectrum beta-lactamases
et al.	et alii or and other people
EU	The European Union
EUCAST	The European Committee on Antimicrobial Susceptibility Testing
F	
FAO	Food and Agriculture Organization of the United Nations
FED	cefepime
FLO	flofenicol
FOX	cefoxitin
G	
GEN	gentamicin
GERMAP	The German Network of Antimicrobial Resistance Surveillance
GfK	Das Veterinärpanel der Gesellschaft für Konsumforschung, The Panel of the Veterinary Association for Consumer Research
H	
h	hours
HA-MRSA	Healthcare-Associated or Hospital-Associated Methicillin-resistant *Staphylococcus aureus*
HRP	Horseradish Peroxidase
I	
IMI	intramammary infection
IMP	imipenem
K	
KAN	kanamycin
kg	kilogram
KPC	Klebsiella Pneumonia Cabapenemase
L	
LA-MRSA	Livestock-Associated Methicillin-resistant *Staphylococcus aureus*
LB	Luria-Bertani
LS	Lauryl Sulfate
LZD	Linezolid
M	
µg	microgram
µl	microliter
ml	milliliter
min	minute
MAC	MacConkey
MABUSE	Medical Antibiotic Use Surveillance and Evaluation
MDR	Multidrug resistance
MHA	Mueller Hinton Agar
MHB	Mueller Hinton Broth
MIC	Minimum inhibitory concentrations
MLST	multilocus sequence typing
MRIJ	The Meusse-Rhine-IJssel
MRSA	Methicillin-resistant *Staphylococcus aureus*
MUP	Mupirocin
N	
n	number of samples

NAL	nalidixic acid
NSAID	non-steroidal antiinflammation drug
NT-MRSA	Non-Typable Methicillin-resistant *Staphylococcus aureus*
O	
OIE	Office international des epizooties (World Organisation for Animal Health)
OXA	oxacillin
P	
PBP	penicillin-binding protein
PCR	Polymerase chain reaction
PFGE	pulsed field gel electrophoresis
PIP	piperacillin
Q	
QUI/DAL	quinupristin/dalfopristin
R	
RDD	recommended daily doses
S	
S	streptomycin
SARI	Surveillance of Antibiotic consumption and Resistance in Intensive care
S. aureus	*Staphylococcus aureus*
SCC	somatic cell count
SPE	spectinomycin
SRB	Swedish Red & White or Swedish Red Breed
SU	sulfonamide
SXT	sulfametoxazole and trimethoprim
T	
t	ton
TET	tetracycline
TIC	ticarcillin
TMP	trimethoprim
TSB	Tryptone Soy Broth
V	
VAN	vancomycin
VCIA	Veterinary Critically Important Antimicrobials
VHIA	Veterinary Highly Important Antimicrobials
VIA	Veterinary Important Antimicrobials
VRE	Vancomycin-resistant enterococci
W	
WHO	World Health Organization
WldO	Wissenschaftliches Institut der AOK (Allgemeine OrtsKrankenkasse), Research Institute of Public health insurance

B. Questionnaire use in this study

Betriebsdaten Datum: …….. Code Nr.: ……

Anzahl Milchkühe: ……	Anzahl Kälber bis 6 Monate: ……	Anzahl Färsen >6 Monate: ……
	Anzahl Kühe laktierend: ……	Anzahl Trockensteher: ……
Milchquote (t / Jahr) … …	Abgänge Kühe / Jahr: ……	

Aufzucht*:	Selbst	Aufzuchtbetrieb gemeinsam mit anderen	
Remontierung:	Aus eigener Nachzucht (%): ……	Über Zukauf (%): ……	

Werden auf dem Betrieb weitere Tierarten gehalten?*	Schweine	Geflügel
	Hunde	Sonstige

**zutreffendes bitte ankreuzen*

Tiergesundheit und Behandlungen

Krankheitskomplex Milchviehherde	**Vorkommen[1]**	**Anwendung von Antibiotika**	**Welche Antibiotika (Handelsnamen)**
Lahmheit (Klauenerkrankungen)	- + ++ +++	Häufig nie	……
Gebärmutterentzündung	- + ++ +++	Häufig nie	……
Schwergeburten	- + ++ +++	Häufig nie	……
Operationen (Labmagenverl., Kaiserschn.)	- + ++ +++	Häufig nie	……
Jungvieh			
Atemwegserkrankungen	- + ++ +++	Häufig nie	Name: …… Gabe mit Milch mittels Injektion
Durchfallerkrankungen	- + ++ +++	Häufig nie	Name: …… Gabe mit Milch mittels Injektion
Nabelentzündungen	- + ++ +++	Häufig nie	Name: …… Gabe mit Milch mittels Injektion
Gelenksentzündungen	- + ++ +++	Häufig nie	Name: …… Gabe mit Milch mittels Injektion

[1] *(-) kommt fast nicht vor, (+) kommt selten vor, (++) kommt regelmäßig vor, (+++) häufig, ist erhebliches Problem*

Fragebogen zum Gesundheitsmanagement

Überwachung der Eutergesundheit

Aus welchem Anlass nehmen Sie Milchproben zu bakteriologischen Untersuchung?[1]	Nach dem Abkalben	Bei hoher MLP-Zellzahl
	Vor dem Trockenstellen	Bei Flocken in der Milch
	Nach der Behandlung	Bei starker Entzündung

[1] *zutreffendes bitte ankreuzen, Mehrfachnennungen möglich*

Behandlung von Euterentzündungen

Wann behandeln Sie Kühe gegen Mastitis?*	Hohe Zellzahlen		Flocken in der Milch		Starke Entzündung	
	Häufig	nie	Häufig	nie	Häufig	nie

Wie viele Tiere werden pro Monat gegen Mastitis behandelt? ca.: ……

Wo stehen die Kühe während der Milchsperre?*			Herde	Krankenstall/-gruppe
Wie oft wird behandelt?*	1-2 Tg	3-4 Tg	>4 Tg	bis keine Flocken mehr vorhanden
Wie wird behandelt?	Das betroffene Viertel in % ……		Alle Viertel in % ……	

Womit werden die akuten Mastitiskühe behandelt? *(Handelsnamen, geschätzter Anteil, keine Trockenstellerl)*	1)……	2)……
	3)……	4)……
	5)……	6)……

Wo werden die Behandlungen dokumentiert?*	Herdenmanagement -programm	Bestandsbuch/ Krankenkartei

Werden alle Kühe unter antibiotischem Schutz trockengestellt?*	Ja	Nein

Nach welchen Kriterien wird über die Art des Trockenstellens entschieden?* (Mehrfachnennungen möglich)	Zellzahl bei MLP	
	Schalmtest	
	Bakteriol. Untersuchung	
	Mastitis in der Laktation	
Mit welchen Präparaten werden die Tiere trockengestellt?	1) ……	2) ……
	3) ……	4) ……

C. Tables and Figures

Table C1. Results of ESBLs producing coliforms and MRSA from bulk tank milk from the 60 dairy farms

Number	Sample ID	ESBLs coliforms	MRSA
1	**01**	***E. coli* (99.9%)**	-
2	**02**	***E. coli* (99.5%)**	-
3	**03**	***E. coli* (99.5%)**	-
4	**04**	-	-
5	**05**	***E. coli* (99.5%)**	-
6	**06**	-	-
7	**07**	-	-
8	**08**	-	-
9	**09**	*Citrobacter braakii* (57.5%) *Citrobacter freundii* (36.9%)	-
10	**10**	-	-
11	**11**	-	-
12	**12**	-	-
13	**13**	*Hafnia alvei* (97.6%)	-
14	**14**	***E. coli* (99.9%)**	-
15	**15**	***E. coli* (99.9%)**	-
16	**16**	*Hafnia alvei* (99.5%)	-
17	**17**	-	-
18	**18**	*Enterobacter cloacae* (99.4%)	-
19	**H01**	-	-
20	**H02**	-	-
21	**H03**	*Enterobacter cloacae* (95.1%)	-
22	**H04**	-	-
23	**H05**	*Enterobacter cloacae* (93.1%)	-
24	**H22**	-	-
25	**H06**	-	-
26	**H07**	***E. coli* (99.8%)**	**MRSA**
27	**H08**	-	-
28	**H09**	-	-
29	**H10**	-	-
30	**H11**	-	-
31	**H13**	*Citrobacter freundii* (99.9%)	-
32	**H14**	-	-
33	**H12**	-	-
34	**H21**	***E. coli* (99.9%)**	-
35	**H23**	-	-
36	**H24**	***E. coli* (99.5%)**	-
37	**H25**	***E. coli* (97.9%)**	-
38	**H26**	*Klebsiella pneumonia* (54.5%)	-
39	**H27**	-	-

Number	Sample ID	ESBLs coliforms	MRSA
40	**H28**	*Enterobacter cloacae* (93.1%)	-
41	**H29**	***E. coli* (99.8%)**	-
42	**H30**	***E. coli* (99.5%)**	-
43	**H31**	-	-
44	**H40**	***E. coli* (99.8%)**	**MRSA**
45	**H15**	-	-
46	**H16**	-	-
47	**H20**	***E. coli* (98.1%)**	-
48	**H17**	***E. coli* (99.8%)**	-
49	**H18**	*Hafnia alvei* (99.9%)	**MRSA**
50	**H19**	***E. coli* (98.1%)**	-
51	**H32**	***E. coli* (97.7%)**	-
52	**H34**	***E. coli* (99.8%)**	**MRSA**
53	**H300**	*Klebsiella pneumonia* (97.6%)	-
54	**H33**	*Hafnia alvei* (92.9%)	-
55	**H35**	-	-
56	**H36**	-	-
57	**H37**	-	-
58	**H38**	*Hafnia alvei* (97.6%)	-
59	**H39**	-	-
60	**H400**	*Hafnia alvei* (82.5%)	-

Table C2. Susceptibility breakpoints for 15 standard antimicrobial agents for enterobacteria from disk diffusion test of ESBLs-producing *E. coli* (CLSI, 2009b)

Antimicrobial agent	Zone of inhibition (mm)		
	resistant	intermediate	sensitive
Ampicillin (10 µg)	≤ 13	14 - 16	≥ 17
Chloramphenicol (30 µg)	≤ 12	13 - 17	≥ 18
Flofenicol (30 µg)	≤ 14	15 - 18	≥ 19
Gentamicin (10 µg)	≤ 12	13 - 14	≥ 15
Kanamycin (30 µg)	≤ 13	14 - 17	≥ 18
Cipofloxacin (5 µg)	≤ 15	16 - 20	≥ 21
Nalidixic acid (30 µg)	≤ 13	14 - 18	≥ 19
Amoxicillin+clavulanic acid (30 µg)	≤ 14	15 - 16	≥ 17
Tetracycline (30 µg)	≤ 11	12 - 14	≥ 15
Streptomycin (10 µg)	≤ 11	12 - 14	≥ 15
Spectinomycin (100 µg)	≤ 10	11 - 13	≥ 14
Sulfamethoxazole (300 µg)	≤ 12	13 - 16	≥ 17
Trimethoprim (5 µg)	≤ 10	11 - 15	≥ 16
Trimethoprim/sulfamethoxazole (25 µg)	≤ 10	11 - 15	≥ 16
Ceftiofur (30 µg)	≤ 17	18 - 20	≥ 21

Table C3. Susceptibility breakpoints for 15 beta-lactams antimicrobial agents from disk diffusion test of ESBLs-producing *E. coli* (CLSI, 2009b)

Antimicrobial agent	Zone of inhibition (mm)		
	resistant	intermediate	sensitive
AMP (10 μg)	≤ 13	14 - 16	≥ 17
CEF (30 μg)	≤ 14	15 - 17	≥ 18
CXM (30 μg)	≤ 14	15 - 22	≥ 23
TIC (75 μg)	≤ 14	15 - 19	≥ 20
PIP (100 μg)	≤ 17	18 - 20	≥ 21
EFT (30 μg)	≤ 17	18 - 20	≥ 21
CRO (30 μg)	14	15 - 17	18
IMP (10 μg)	≤ 13	14 - 15	≥ 16
CTX (30 μg)	≤ 14	15 - 22	≥ 23
AMC (30 μg)	≤ 14	15 - 16	≥ 17
CAZ (30 μg)	14	15 - 17	18
AZM (30 μg)	≤ 15	16 - 21	≥ 22
CPD (10 μg)	≤ 17	18 - 20	≥ 21
FOX (30 μg)	≤ 14	15 - 17	≥ 18
FEP (30 μg)	≤ 14	15 - 27	≥ 18

AMP – Ampicillin, CEF – Cephalothin, CXM – Cefuroxime,TIC – Ticarcillin, PIP – Piperacillin, EFT – Ceftiofur, CRO – Ceftriaxone, IMP – Imipenem, CTX – Cefotaxime, AMC - Amoxicillin+Clavulanic acid, CAZ – Ceftazidime, AZM – Aztreonam , CPD – Cefpodoxime , FOX – Cefoxitin , FEP – Cefepime

Table C4. Susceptibility breakpoints of 13 antimicrobial agents used in the antimicrobial susceptibility testing of MRSA

Antimicrobial agent	Cut-off ≤ µg/ml	concentrations		Reference[a]
		Minimum µg/ml	Maximum µg/ml	
Gentamicin	2	0.5	64	EUCAST
Kanamycin	8	8	128	EUCAST
Chloramphenicol	16	2	256	EUCAST
Ciprofloxacin	1	0.5	64	EUCAST
Oxacillin	2	0.5	8	EUCAST
Trimethoprim/sulfamethoxazole	0.5	0.25/4.8	16/304	EUCAST
Tetracycline	1	1	64	EUCAST
Clindamycin	0.25	0.25	32	EUCAST
Erythromycin	1	0.12	16	EUCAST
Mupirocin	1	1	16	EUCAST
Linezolid	4	1	16	EUCAST
Quinupristin/Dalfopristin	1	0.5	8	EUCAST
Vancomycin	2	2	32	EUCAST

[a] Evaluation of resistance was based on epidemiological cut-off values published by the European committee for antimicrobial susceptibility testing for MRSA and Staph. aureus (www.eucast.org).

Table C5. Explanation of gene encoding for toxic shock syndrome toxin, leukocidin, and haemolysin by DNA micro array analysis

Gene	Explanation
***tst*-1**	Toxic shock syndrome Toxin
***tst*-RF122**	Toxic shock syndrome Toxin, allele from bovine strains
PVL	Pantone-Valentine Leukocidin
***luk*M/lukF-P83**	Bovine Leukocidin
***luk*F**	Haemolysin Gamma, Component B
***luk*S**	Haemolysin Gamma, Component C
***luk*S-ST22+ST45**	Haemolysin Gamma, Component C, allele from ST22 and ST45
***hlg*A**	Haemolysin Gamma, Component A
***luk*D**	Leukocidin D Component
***luk*E**	Leukocidin E Component
***luk*X**	Leukocidin/Haemolysin Toxin Family Protein
***luk*Y-var1**	Leukocidin/Haemolysin Toxin Family Protein
***luk*Y-var2**	Leukocidin/Haemolysin Toxin Family Protein, allele from MRSA252
hl	Hypothetical Protein similar to Haemolysin
hla	Haemolysin Alpha (Alpha Toxin)
hld	Haemolysin Delta (Amphiphylic Membrane Toxin)
***hl*III**	Putative Haemolysin III
***hl*_III_Other than RF122**	Putative Haemolysin III (Other than RF122)
hlb	Haemolysin Beta (Phospholipase C)
Un-truncated *hlb*	Haemolysin Beta (Phospholipase C/ un-truncated)

Table C6. Explanation of gene encoding for antimicrobial resistance by DNA micro array analysis

gene	Explanation
***mec*A**	Methicillin, Oxacillin and all Beta-Lactams, defining **MRSA**
***bla*Z**	Beta-Lactamase
***bla*I**	Beta-Lactamase Repressor (Regulatory Protein)
***bla*R**	Beta-Lactamase Regulatory Protein
***erm*A**	Macrolide, Lincosamide, Streptogramin
***erm*B**	Macrolide, Lincosamide, Streptogramin
***erm*C**	Macrolide, Lincosamide, Streptogramin
***lin*A**	Lincosamide
***msr*A**	Macrolide
***mef*A**	Macrolide
***mpb*BM**	Macrolide
***vat*A**	Streptogramin
***vat*B**	Streptogramin
vga	Streptogramin
***vga*A**	Streptogramin
vab	Streptogramin
***aac*A-*aph*D**	Aminoglycoside (Gentamicin, Tobramycin)
***aad*D**	Aminoglycoside (Tobramycin, Neomycin)
***aph*A**	Aminoglycoside (Kanamycin, Neomycin)
sat	Streptotricin
***dfr*A**	Trimethoprim
Far	Fusidic acid
Q6GD50	Putative Fusidic acid Resistance Protein
***mup*R**	Mupirocin
***tet*K**	Tetracycline
***tet*M**	Tetracycline
***tet*Efflux**	Tetracycline Efflux Protein (Putative Transport Protein)
cat	Chloramphenicol
***fex*A**	Chloramphenicol
cfr	Phenicols, Lincosamide, Oxazolidinones (Linezolid), Pleuromutilins, Streptogramin A
***fos*B**	Putative Marker for Fosfomycin, Bleomycin
***van*A**	Vancomycin
***van*B**	Vancomycin
***van*Z**	Vancomycin
Mercury resistance locus	Mercury resistance operon
***qac*A**	Unspecific efflux pump
***qac*C**	Unspecific efflux pump

Table C7. Explanation of gene encoding for enterotoxins by DNA micro array analysis

Gene	Explanation
***ent*A**	Enterotoxin A
***ent*A-320E**	Enterotoxin A, allele from 320E
***ent*A-N315**	Enterotoxin A, allele from N315
***ent*B**	Enterotoxin B
***ent*C**	Enterotoxin C
***ent*CM14**	Enterotoxin –like protein (ORF CM14 of U10927.2)
***ent*D**	Enterotoxin D
***ent*E**	Enterotoxin E
***ent*G**	Enterotoxin G
***ent*H**	Enterotoxin H
***ent*I**	Enterotoxin I
***ent*J**	Enterotoxin J
***ent*K**	Enterotoxin K
***ent*L**	Enterotoxin L
***ent*M**	Enterotoxin M
***ent*N**	Enterotoxin N
***ent*N_1**	Enterotoxin N-other than RF122
***ent*O**	Enterotoxin O
***ent*Q**	Enterotoxin Q
***ent*R**	Enterotoxin R
***ent*U**	Enterotoxin U
***egc*-cluster**	Enterotoxins seg/sei/sem/sen/seo/seu

Fig C1. Presence of genes encoding staphylococcal enterotoxins among 36 MRSA strains by DNA microarray analysis

sample ID	entA	entA-320E	entA-N315	entB	entC	entCM14	entD	entE	entG	entH	entI	entJ	entK	entL	entM	entN	entN_1	entO	entQ	entR	entU	egc-cluster
09S-160	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
09S-161	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
09S-162	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
09S-196	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
09S-531	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
09S-534	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
09S-535	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
09S-627	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
09S-629	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
09S-1065	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
09S-1066	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
09S-1076	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
09S-1088	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
09S-1380	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
09S-1386	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
09S-1391	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
09S-1659	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
09S-1770	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
09S-1772	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
09S-1774	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
09S-1776	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
09S-2339	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
09S-2376	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
10S-423	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
10S-428	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
10S-500	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
10S-969	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
10S-1200	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
10S-1237	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
10S-1253	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
10S-1399	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
H07-1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
H07-2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
H18	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
H34	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
H40	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0

Fig C2. Presence of genes encoding toxic shock syndrome toxins, leukocidins, and hemolysins among 36 MRSA strains by DNA microarray analysis

sample ID	tst-1	tst-RF122	PVL	lukM/lukF-P83	lukF	lukS	lukS-ST22+ST45	hlgA	lukD	lukE	lukX	lukY-var1	lukY-var2	hl	hla	hld	hlIII	hl_III_other than RF122	hhlb	truncated hlb
09S-160	0	0	0	0	1	1	0	1	0	0	1	1	0	1	1	1	1	1	0	1
09S-161	0	0	0	0	1	1	0	1	0	0	1	1	0	1	1	1	1	1	0	1
09S-162	0	0	0	0	1	1	0	1	0	0	1	1	0	1	1	1	1	1	0	1
09S-196	0	0	0	0	1	1	0	1	0	0	1	1	0	1	1	1	1	1	0	1
09S-531	0	0	0	0	1	1	0	1	0	0	1	1	0	1	1	1	1	1	0	1
09S-534	0	0	0	0	1	1	0	1	0	0	1	1	0	1	1	1	1	1	0	1
09S-535	0	0	0	0	1	1	0	1	0	0	1	1	0	1	1	1	1	1	0	1
09S-627	0	0	0	0	1	1	0	1	0	0	1	1	0	1	1	1	1	1	0	1
09S-629	0	0	0	0	1	1	0	1	0	0	1	1	0	1	1	1	1	1	0	1
09S-1065	0	0	0	0	1	1	0	1	0	0	1	1	0	1	1	1	1	1	0	1
09S-1066	0	0	0	0	1	1	0	1	0	0	1	1	0	1	1	1	1	1	0	1
09S-1076	0	0	0	0	1	1	1/0	1	0	0	1	1	0	1	1	1	1	1	0	1
09S-1088	0	0	0	0	1	1	1/0	1	0	0	1	1	0	1	1	1	1	1	0	1
09S-1380	0	0	0	0	1	1	0	1	0	0	1	1	0	1	1	1	1	1	0	1
09S-1386	0	0	0	0	1	1	0	1	0	0	1	1	0	1	1	1	1	1	0	1
09S-1391	0	0	0	0	1	1	0	1	0	0	1	1	0	1	1	1	1	1	0	1
09S-1659	0	0	0	0	1	1	0	1	0	0	1	1	0	1	1	1	1	1	0	1
09S-1770	0	0	0	0	1	1	0	1	0	0	1	1	0	1	1	1	1	1	0	1
09S-1772	0	0	0	0	1	1	1/0	1	0	0	1	1	0	1	1	1	1	1	0	1
09S-1774	0	0	0	0	1	1	0	1	0	0	1	1	0	1	1	1	1	1	0	1
09S-1776	0	0	0	0	1	1	0	1	0	0	1	1	0	1	1	1	1	1	0	1
09S-2339	0	0	0	0	1	1	1/0	1	0	0	1	1	0	1	1	1	1	1	0	1
09S-2376	0	0	0	0	1	1	0	1	0	0	1	1	0	1	1	1	1	1	0	1
10S-423	0	0	0	0	1	1	0	1	0	0	1	1	0	1	1	1	1	1	0	1
10S-428	0	0	0	0	1	1	1/0	1	0	0	1	1	0	1	1	1	1	1	0	1
10S-500	0	0	0	0	1	1	0	1	0	0	1	1	0	1	1	1	1	1	0	1
10S-969	0	0	0	0	1	1	0	1	0	0	1	1	0	1	1	1	1	1	0	1
10S-1200	0	0	0	0	1	1	1/0	1	0	0	1	1	0	1	1	1	1	1	0	1
10S-1237	0	0	0	0	1	1	1/0	1	0	0	1	1	0	1	1	1	1	1	0	1
10S-1253	0	0	0	0	1	1	1	1	0	0	1	1	0	1	1	1	1	1	0	1
10S-1399	0	0	0	0	1	1	1	1	0	0	1	1	0	1	1	1	1	1	0	1
H07-1	0	0	0	0	1	1	1/0	1	0	0	1	1	0	1	1	1	1	1	0	1
H07-2	0	0	0	0	1	1	1/0	1	0	0	1	1	0	1	1	1	1	1	0	1
H18	0	0	0	0	1	1	1/0	1	0	0	1	1	0	1	1	1	1	1	0	1
H34	0	0	0	0	1	1	0	1	0	0	1	1	0	1	1	1	1	1	0	1
H40	0	0	0	0	1	1	0	1	0	0	1	1	0	1	1	1	1	1	0	1

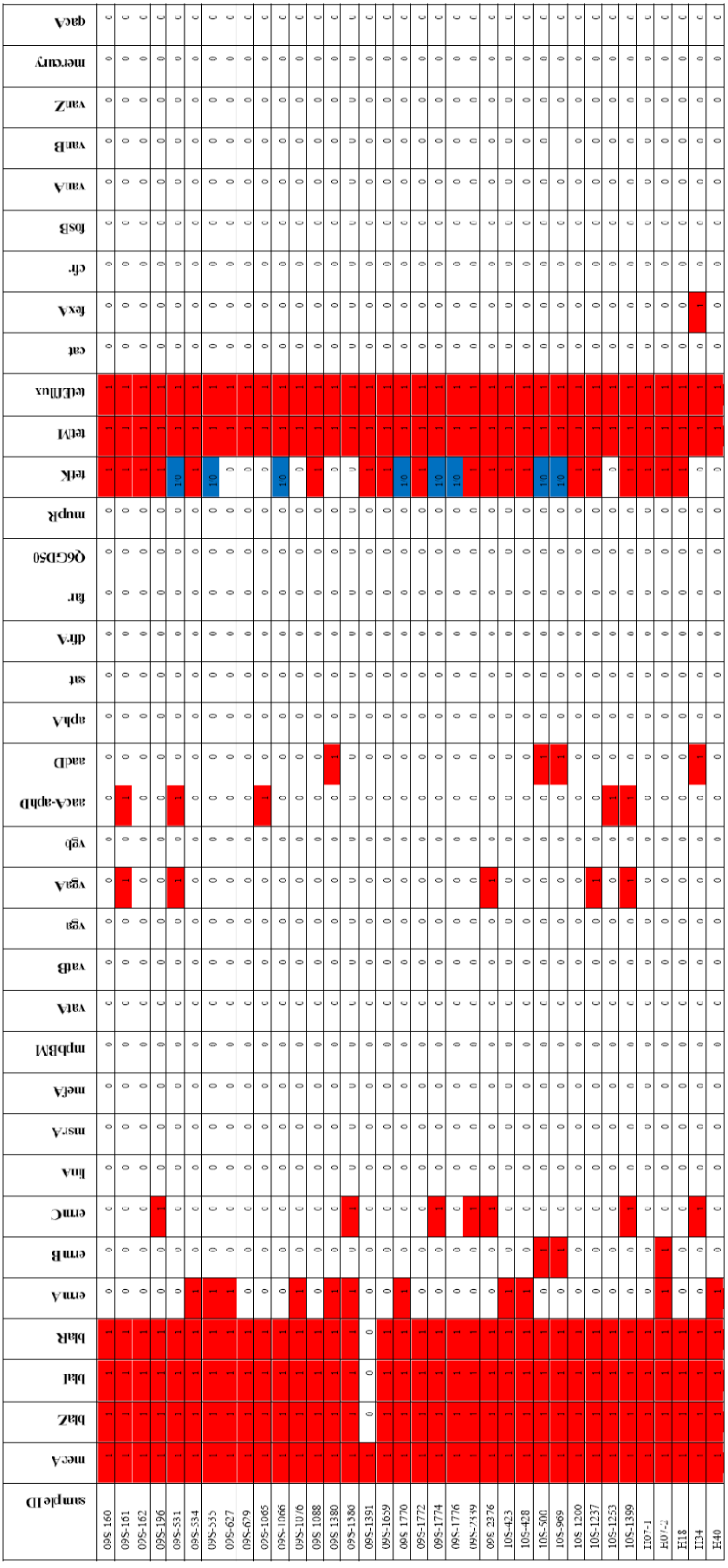

Fig C3. Presence of genes encoding antimicrobial resistance among 36 MRSA strains by DNA microarray analysis

Chapter 10
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Selbständigkeitserklärung

Hiermit bestätige ich, dass ich die vorliegende Arbeit selbständig angefertigt habe. Ich versichere, dass ich ausschlieβlich die angegebenen Quellen und Hilfen in Anspruch genommen habe.

Berlin, 20. July 2011 Khwanchai Kreausukon